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DOKUZ EYLÜL UNIVERSITY
IZMIR INTERNATIONAL
BIOMEDICINE AND GENOME
INSTITUTE

THE MOLECULAR MECHANISM OF THE SWITCH OF ABCG1 DEFICIENT MACROPHAGES TO A TUMOR FIGHTING M1 PHENOTYPE

MELTEM ALTUNAY

MOLECULAR BIOLOGY AND GENETICS

MASTER'S THESIS

IZMIR-2019

THESIS ID: DEU.IBG.MSc/ 2016850016

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ASSOC. PROF. DUYGU SAG

This Study is supported by TUBITAK-EU 115C074,
DEU 2017.KB.SAG.011, L'Oreal UNESCO

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Dokuz Eylül University Izmir International Biomedicine and Genome Institute

Genom Bilimleri ve Moleküler Biyoteknoloji Anabilim Dalı, Yüksek Lisans programı öğrencisi Meltem ALTUNAY '**ABCG1 GENİ SUSTURULMUŞ MAKROFAJLARIN TÜMÖRLE SAVAŞAN M1 FENOTİPİNE DÖNÜŞMESİNİN MOLEKÜLER MEKANİZMASI**' konulu Yüksek Lisans tezini 05.07.2019 tarihinde başarılı olarak tamamlamıştır.

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ABBREVIATIONS

ABCG1: ATP-Binding Casette Transporter G1

AMPK: AMP- Activated Protein Kinase

LPS: Lipopolysaccharide

IFNγ: Interferon Gamma

IL-4: Interleukin 4

TNFα: Tumor necrosis factor alpha

TLR: Toll Like Receptor

Th1: T helper 1

Th2: T helper 2

PRR: Pattern Recognition Receptor

IL-12: Interleukin 12

IL-13: Interleukin 13

STAT1: Signal Transducer and Activator of Transcription 1

STAT6: Signal Transducer and Activator of Transcription 6

CCL5: Chemokine (C-C motif) ligand 5

iNOS: Inducible nitric oxide synthase

Cox-2: cyclooxygenase-2

Arg-1: Arginase 1

Mrc1: Mannose Receptor C-Type 1

Fizz1: Resistin-like molecule alpha1

ABCA1: ATP- Binding Casette Transporter A1

HDL: High Density Lipoprotein

LXR: Liver X Receptor

Thr: Threonine

Ser: Serine

LKB1: Liver Kinase B1

CaMKKβ: Calcium/Calmodulin-Dependent Protein Kinase Kinase 2

NF-kB: Nuclear Factor kappa B

IL-6: Interleukin 6

IL-8: Interleukin 8

AICAR: 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside

mTOR: Mammalian Target of Rapamycin

mTORC: Mammalian Target of Rapamycin Complex

PDK1: Pyruvate Dehydrogenase Kinase 1

PI3K: Phosphoinositide 3-kinase

DN-AMPK: Dominant Negative AMPK

CA-AMPK: Constutively Active AMPK

M-CSF: Macrophage Colony Stimulating Factor

BSA: Bovine Serum Albumine

ELISA: Enzyme-Linked ImmunoSorbent Assay

PBS: Phosphate Buffered Saline

FBS: Fetal bovine serum

ACKNOWLEDGMENT

First of all, I would like to convey my sincere respect and love to my advisor Duygu Sağ. I am grateful that during my three years of scientific work, she showed me the basics of scientific thinking and included me to her own laboratory. It is great chance for me to have an opportunity to work in her lab. I thank her again for the support that she gave me and for the free working conditions that she created for us. If you have free working conditions in the laboratory than you could take big steps in science and Sağ Lab is a great home to take those steps.

I would like to thank Duygu Ünüvar Purcu from my lab, because of her support and for help on troubleshooting of my experiments. And she is like a real sister to me. I am grateful that she still enlightens me in life and science. Sinem Günalp is my other lab sister and friend who I want to thank. I want to thank her for helping me with every step of the way in designing my experiments. I want to thank Resul, especially for helping me with mice experiments. I would like to thank Aslı, the newest member of our Lab, for helping me to organize my thesis. I would like to thank Göksu Helvacı, an intern student who has been working in our lab for a long time and who is the youngest member of our lab, for his contribution and good questions on my work. I would like to thank Gizem Eylem Başlar, the first intern student of our lab, for her help during the period that she worked in our lab.

I also would like to thank Gerhard Wingender for his help about how we should intreprete the science. To have journal club with him and his lab was a great experience for me. I would like to thank each of his lab members for their support and help. Especially our scientific discussions with Zeynep Özge Ayyıldız contributed to the enlightenment of my path. I also would like to thank Fatma Zehra Hapil for sharing her knowledge with us.

I want to thank to Zehra Tavşan from Ayar-Kayalı lab because of her help with Western blot. And also I would like to thank our institute to create us such peaceful and comfortable space to work in.

Lastly, I would like to thank my family for their endless love and being always supportive and always by my side. They are heroes of my life, I always feel their existence

and their help in every step of my life and I am really grateful for that. During this period, I cannot thank enough to my sister and also my home mate Mine Altunay. I am grateful to have a sister like her who has supported me to complete this long and tough journey.

THE MOLECULAR MECHANISM OF THE SWITCH OF ABCG1 DEFICIENT MACROPHAGES TO A TUMOR FIGHTING M1 PHENOTYPE

ABSTRACT

Macrophages that are major players of tumor immunity, are divided into two subgroups as M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages. In general, M1 macrophages are potent tumor-fighting cells, whereas M2 macrophages display protumoral functions. ATP-binding Cassette Transporter G1 (ABCG1) promotes cholesterol efflux from cells and regulates intracellular cholesterol homeostasis. We have recently shown that in the absence of ABCG1, macrophages shift from a tumor-promoting M2 phenotype to a tumor-fighting M1 phenotype within the tumor and suppress bladder cancer growth in vivo. The molecular mechanism through which ABCG1-deficiency shifts macrophages to a tumor fighting M1 phenotype is not known. To address the molecular mechanism, we analysed cell signalling in bone marrow-derived macrophages from Abcg1-/- mice. Compared to WT control, Abcg1-/- macrophages displayed reduced levels of Akt activation at basal level and after stimulation with the M1 signal LPS/IFNg or the M2 signal IL4 at different time points. Furthermore, in concordiance with the M1 bias, while Abcg1-/- macrophages stimulated with LPS/IFNg produced more TNFα compared to WT macrophages, after treatment with an Akt activator(SC79) the TNFa-production of Abcg1-/- and WT macrophages was comparable. These data suggest that the M1 bias of Abcg1-/- macrophages is mediated through the Akt signalling pathway. These findings not only deepen our mechanistic understanding of the M1/M2 switch in macrophages, but have the potential to open up new immunotherapeutic approaches for the treatment of cancer.

KEYWORDS; ABCG1, Akt, LPS, IFNg, TNFa, M1 macrophages, M2 macrophages

ABCG1 GENİ SUSTURULMUŞ MAKROFAJLARIN TÜMÖRLE SAVAŞAN M1 FENOTİPİNE DÖNÜŞMESİNİN MOLEKÜLER MEKANİZMASI

ÖZET

Tümör immünolojisinin ana oyuncuları olan makrofajlar, M1 (pro-inflamatuar) ve M2 (anti- inflamatuar) makrofajlar olmak üzere iki alt gruba ayrılır. Genel olarak, M1 makrofajları güçlü tümörle savaşan hücrelerdir, oysa M2 makrofajları protumoral fonksiyonlar sergiler. ATP bağlayıcı Kaset Taşıyıcı G1 (ABCG1), hücrelerden kolesterol akışını arttırır ve hücre içi kolesterol homeostazını düzenler. Son zamanlarda, ABCG1'in yokluğunun, tümör içindeki makrofaj fonksiyonunu modüle ederek farelerde tümör büyümesini inhibe ettiğini gösterdik. ABCG1'in yokluğunda makrofajlar, tümör içinde M2 fenotipinden M1 fenotipine geçer ve tümör hücrelerini doğrudan öldürür. ABCG1 eksikliğinin, makrofajları M1 fenotipine dönüştürmesinin moleküler mekanizması bilinmemektedir. Moleküler mekanizmayı ele almak için Abcg1 -/- farelerinin kemik iliğinden üretilen makrofajlarda hücre sinyalizasyonunu analiz ettik. Bu makrofajlar, LPS / IFNg veya IL4 ile uyarıldıktan sonra düşük Akt aktivasyon seviyeleri sergilemiştir. Ek olarak, LPS / IFNg ile uyarılan Abcg1 -/- makrofajları WT makrofajlarına kıyasla daha fazla TNFa üretirken, bir Akt aktivatörü ile muameleden sonra Abcg1 -/- ve WT makrofajlarının TNFa üretimi benzerdir. Bu veriler Abcg1 -/- makrofajların M1 yanlılığının Akt sinyal yolağı yoluyla yönetildiğini göstermektedir. Bu bulgular yalnızca makrofajlardaki M1/M2 geçişi hakkındaki mekanik anlayışımızı derinleştirmekle kalmaz, aynı zamanda kanser tedavisi için yeni immünoterapötik yaklaşımlar elde etme potansiyeline de sahiptir.

ANAHTAR KELİMELER; ABCG1, Akt, LPS, IFNg, TNFa, M1 makrofajlar, M2 makrofajlar

1. INTRODUCTION AND AIM

Macrophages are innate immune cells that have an important role in first defense of the body. Macrophages have different roles in different conditions. They either could start inflammation process and eliminate the pathogens or they could initiate tissue healing process. There are two types of macrophages such as M1 (classically activated) macrophages and M2 (alternatively activated) macrophages. M1 macrophages play a role in inflammation and tumor suppression and M2 macrophages play a role in tissue healing and tumor progression. In the tumor microenvironment macrophages have M2- like features. It is known that in different tumor types such as MB49 Bladder carcinoma and B16-F1 melanoma, if ABCG1 protein is deficient, macrophages exhibit a shift towards an M1 phenotype. It has been shown in the same study that this shift occurs with an intrinsic mechanism in macrophages. However, the cause of this shift in the absence of ABCG1 is unknown. The aim of this study is to elucidate the underlying mechanism of the shift towards an M1 phenotype in ABCG1 deficient macrophages.

To address the molecular mechanism, we analysed AMPK and Akt signalling in bone marrow-derived macrophages (BMDMs) from Abcg1-/- and WT control mice. BMDMs were stimulated either with LPS+IFNγ for M1 polarization or with IL-4 for M2a polarization at different time points. Then p-AMPK and p-Akt levels were analyzed by Western blot in both WT and Abcg1 -/- M1 and M2 macrophages. Moreover, macrophages were pre-treated with SC-79 for 2 hours (Akt activator) and then with LPS+IFNγ overnight for M1 polarization and TNFα production was analysed by ELISA.

The results of this study showed that Akt is one of the signaling molecules that plays a role in the shift of the macrophages towards an M1 phenotype in Abcg1 -/- macrophages.

H1: We hypothesize that increased accumulation of cholesterol and/or -derivatives in macrophages in the absence of ABCG1 causes inhibition of Akt1, which polarizes these macrophages to a tumor-fighting M1 phenotype.

2. GENERAL INFORMATION

2.1. Macrophages

2.1.1. Macrophages and Their Role in Immunity

Macrophages are innate immune cells, the first defenders of our body against invaders such as pathogens (1). Macrophages could be divided into two groups as tissue resident macrophages and bone marrow derived macrophages (2). Previously, macrophages were considered as they originated from bone marrow monocytes, however recent studies have shown that there are differences between bone marrow derived macrophages and tissue resident macrophage origins (3).Yolk sac give rise to tissue resident macrophages during embryonic development (4). Tissue resident macrophages have self-renewal properties in adults (5).Monocytes give rise to bone marrow-derived macrophages. Monocytes that exit bone marrow and enter circulation differentiate into macrophages when they enter tissues (2).

Macrophages have role in wound healing, phagocytosis and apoptotic clearance. They also provide homeostasis in tissues. (2). Macrophages recognize pathogens via pattern recognition receptors (PRRs), trigger innate immune system further to eliminate them. Also, they trigger adaptive immunity via presenting the pathogen associated antigens (6,7).

In the case of inflammation in tissue sites, first tissue resident macrophages are the main players. They recognize the pathogens and by secreting chemokines they call bone marrow derived macrophages to help fight with the infection(8). After elimination of infection high percentage of bone marrow derived macrophages die and small amount of them could reside in the tissues(8).

2.1.2. Macrophage Polarization

Macrophages are polarized to M1 (classically activated or pro-inflammatory) or M2 (alternatively activated or anti-inflammatory) type macrophages with stimulation via different cytokines or chemokines (9). There are different types of T helper cells, and they release different cytokines. For example, while T helper 1 (Th1) cells produce IFN-g, T helper 2 (Th2) cells release IL-4 (10) and these molecules that are released from Th cells provide M1 and M2 polarization, respectively. Pro- inflammatory M1 macrophages eliminate the pathogens or scavenge the cell debris(11). On the other hand, anti-inflammatory M2 macrophages have a role in healing process of the wound (11) Macrophages differentiate into M1 phenotype when they encounter with a pathogen or through toll like receptor (TLR) signaling(8). M1 type pro-inflammatory macrophages are activated via IFNγ and LPS and have microbicidal features(12). As a result of activation, they secrete IL-12 -a pro-inflammatory cytokine- and also trigger IL-12 mediated T-helper 1 response(12). M2 macrophages however, trigger T helper 2 response and have a role in resolution of inflammation (12) and they also have role in tissue repair and pathogen clearance(13). Stimulating macrophages via IFNγ or LPS leads to M1 polarization through the STAT1 pathway; on the contrary, stimulation via IL-4 and/or IL-13 leads to M2 polarization through STAT6 signalling (14). TNFα, Cox-2, CCL5, and iNOS are associated with M1 type macrophage polarization and their expression levels are reduced via STAT6 activation. However, Arg-1, Mrc1, Fizz1 and PPAR-γ are associated with with M2 type polarization and their expression is induced via STAT6 activation (1).

There are 3 types of M2 macrophages, IL-4 and/or IL-13 drives macrophages towards M2a, macrophages exhibit M2b differentiation when stimulated with IL-1β and LPS(10). Lastly, macrophages that are stimulated with IL-10 or TGF-β show M2c differentiation(10).

M1 and M2 macrophages differ in the case of usage of Arginine metabolism(15). M1 macrophages use iNOS2 enzyme and they form NO from arginine(15), on the other hand M2a and M2c macrophages use arginine to produce urea and ornithine by Arg-

1(15,16). These products have role in cell growth and division(15). M2a and M2c macrophages have a role in wound healing(17). M2b macrophages have similarities with M1 macrophages in the case of production of inflammatory cytokines; however, at the same time they produce high levels of IL-10, and they provide protection against LPS toxicity(10,16).

2.1.3. Macrophages in Tumors

Tumor associated macrophages (TAMs) are the macrophages in the tumor microenvironment that support tumor growth(18). Macrophages in the tumor area shift from classically activated M1 macrophages (anti tumoral) to alternatively activated M2 (pro-tumoral) phenotype (18). TAMs are characterized according to some of their features such as tumor progression, angiogenesis and they have M2-like features(19).

M1 type macrophages play a role in tumour suppression and prevent tumour growth. M1 macrophages have anti-tumoral properties by producing TNFα and IL-1β and they can also produce NO. These molecules increase cytotoxicity of tumor cells(20). Also, in the tumour microenvironment macrophages encounter with molecules that are released by tumour cells which differentiate the macrophages to M2 phenotype (21).

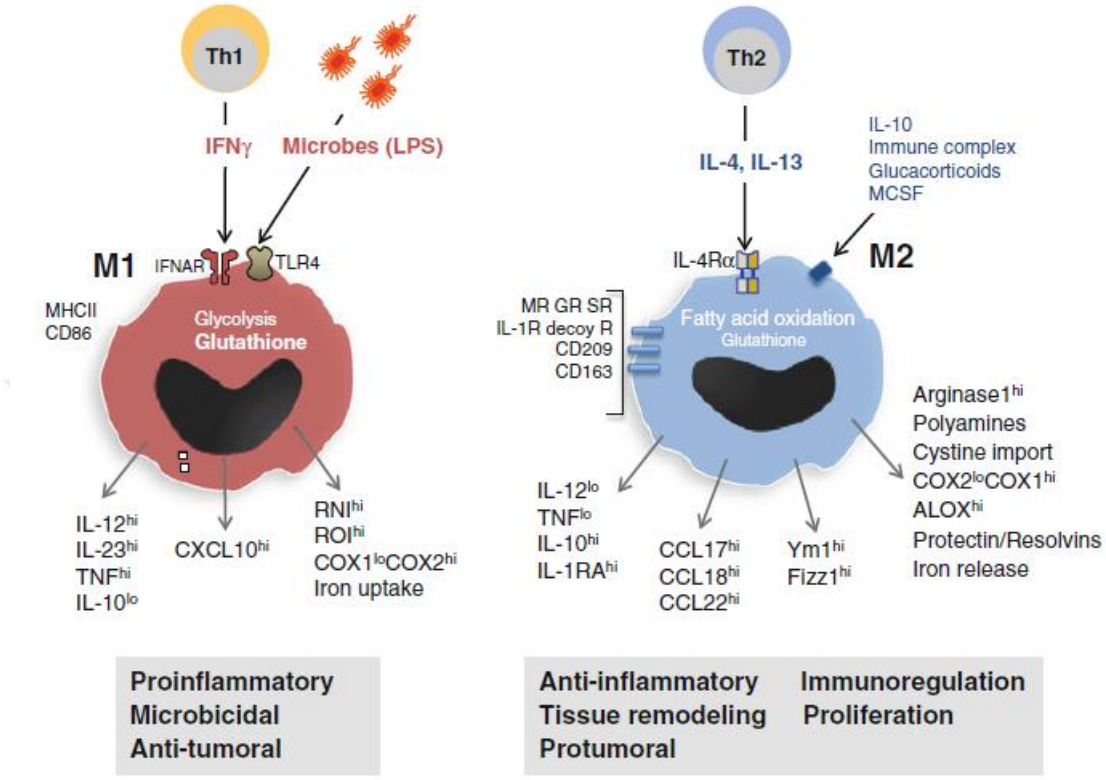


Figure 1. **Role of macrophages in immunity.** Macrophage polarization and the role of macrophages under different conditions (adapted from Biswas et al., 2012).

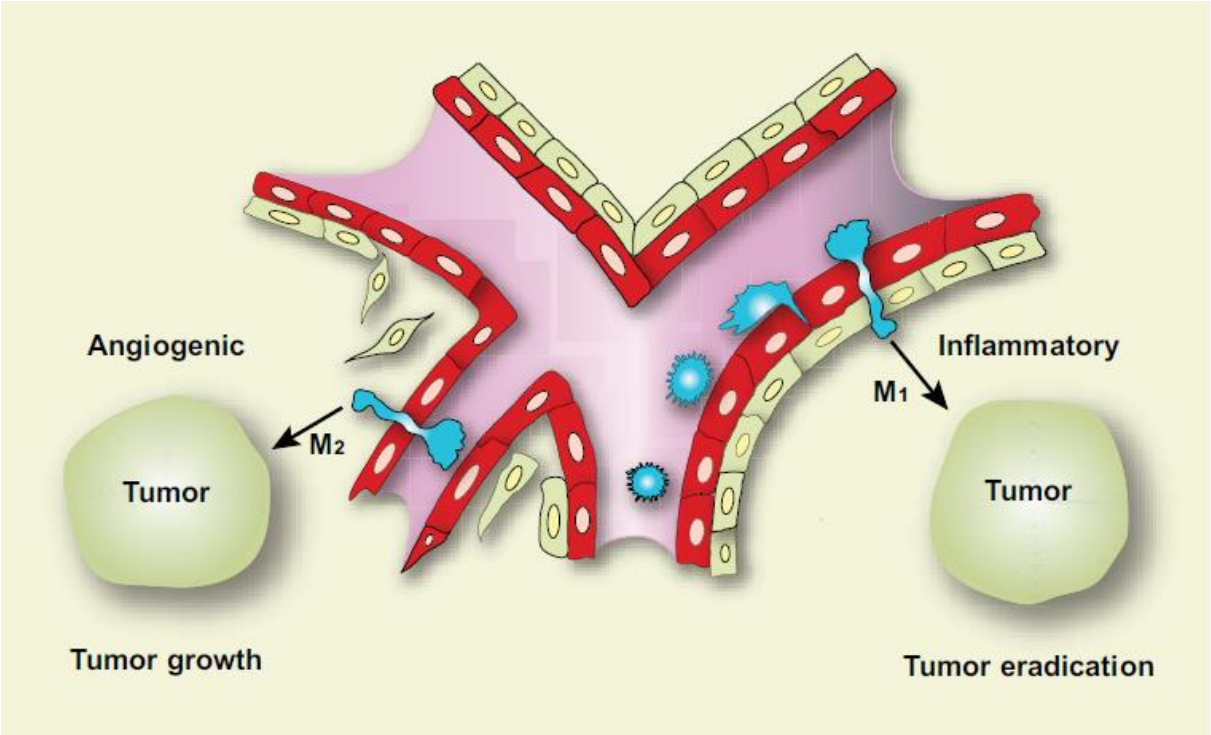


Figure 2. Role of M1 and M2 macrophages in tumor microenvironment. M1 and M2 macrophages in different microenvironments (adapted from Lamagna et al., (2006).

2.2. ABC Transporter G1 Protein (ABCG1)

2.2.1. ABCG1 and cholesterol efflux in macrophages

ABCG1 is a member of the ATP-Binding Cassette (ABC) transporter family. ABC transporters are membrane proteins that export different molecules like lipids, bile salts, and peptides out of the cell(22). There are 48 genes in humans which are responsible for the production of ABC proteins (22). In eukaryotes, these transporters only export molecules across the membrane, however in prokaryotes, they can also import different molecules inside the cell.

ABC transporters use the energy derived from the hydrolysis of ATP, to provide substrate transmission across the membrane (23). They are divided into seven families and named from A to G. ABCA members are responsible for lipid efflux, B members for drug transport, C members could function as channels, and they have a role in chloride ion efflux, and G transporters responsible from cholesterol efflux (24).

Mouse studies have shown that ABCA1, ABCG1 transporters are responsible for cholesterol efflux (25). Earlier studies emphasized the importance of ABCA1 and ABCG1 transporters in decreasing foam cell formation, atherosclerosis and inflammation (26).

Reverse cholesterol transport is the first step of exporting the cholesterol from cells and tissues through high density lipoproteins (HDL) (27). It has been found that ABCG1 is responsible for cholesterol efflux to HDL molecules in macrophages, and ABCG1 deficiency prevents cholesterol efflux to HDL (28). Activation of Liver X receptor (LXR) causes cholesterol efflux by ABCG1 (29, 30) and also has a regulatory role on transcriptional expression of ABCG1 (26).

2.2.2. ABCG1 and macrophage function

It has been shown that the body mass of WT and ABCG1 -/- mice that were fed with chow diet were comparable; however, when they were fed with high fat diet,

ABCG1-/- mice have shown lipid accumulation in lungs and liver(31). The lipid accumulation in lungs was mostly seen in lung tissue resident macrophages (31).

It has been demonstrated in a study that in hypercholesterolomic Abcg1-/- mice atherosclerotic lesions were smaller and the pro-apoptotic gene expression of macrophages was increased compared to control mice (32). In different atherosclerosis studies it has been shown that ABCG1 has protective role by providing cholesterol efflux from macrophages and thereby it prevents foam cell formation (33). It has been concluded that different results of protective effect of ABCG1 in atherosclerosis might be related with stage of the disease(33).

A study has shown that when Abcg1 -/- mice were fed with chow diet, they showed increased accumulation of macrophages and lymphocytes in the lungs at the age of 6-8 months(34). Moreover, Abcg1 -/- mice showed signs of inflammation (34). Parallel to this study, it has been shown in a different study that alveolar macrophages of Abcg1 -/- mice showed increased inflammatory cytokine production (35).

We have previously shown that, ABCG1 -/- mice fed a high fat diet showed suppressed bladder carcinoma and melanoma growth compared to WT mice(36). The reduction in tumor growth in ABCG1-/- mice was associated with the phenotypic shift of macrophages from tumor-promoting M2 to tumor-fighting M1 in the tumor microenvironment(36). In vitro analysis of bone marrow-derived ABCG1-/- macrophages revealed that these macrophages have M1 bias in a cell intrinsic manner (36). The molecular mechanism of this effect is unknown. Hence, the aim of this study is to illuminate the signaling molecules that have a role in the M1 shift in ABCG1 deficiency.

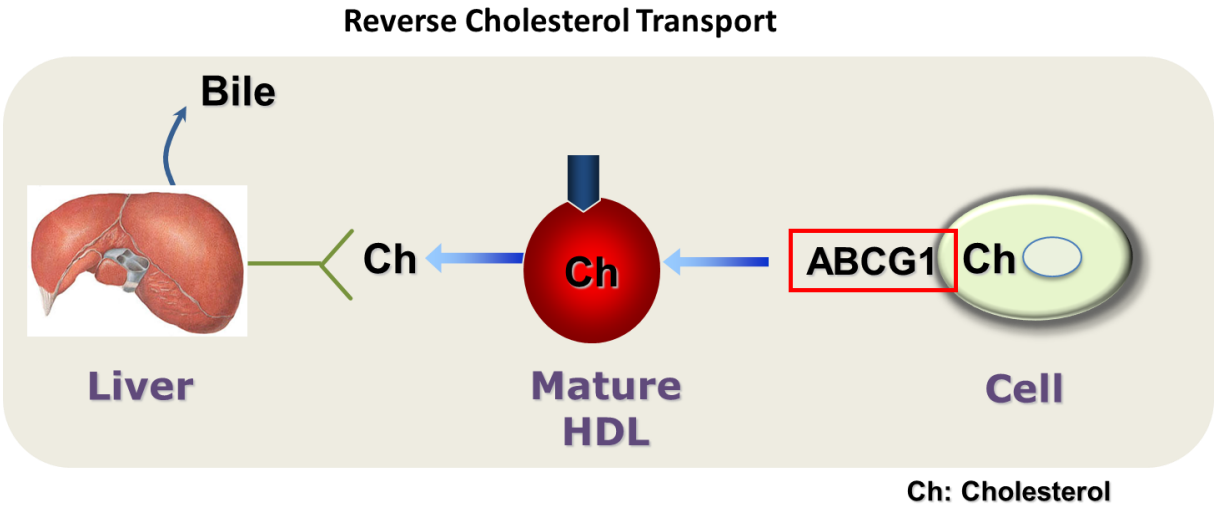


Figure 3. Cholesterol transport by ABCG1. Reverse cholesterol transport from cells by ABCG1 transporter protein.

2.3. AMP Activated Protein Kinase (AMPK)

2.3.1. Definition and the Role of AMPK

AMP activated protein kinase (AMPK) is a serine/threonine kinase that regulates energy metabolism in the cell(37). When the AMP/ATP ratio is high, AMPK is activated to suppress ATP-consuming anabolic pathways(37). In contrast, when the AMP/ATP ratio is low, AMPK is inhibited for the activation of ATP-producing catabolic pathways (37).

AMPK contains catalytic α subunit and regulatory β and γ subunits (38). Homologs of all these three subunits have been found in different eukaryotic species (37).

AMP binding to the regulatory γ subunit causes allosteric activation of AMPK, making the catalytic α subunit available for the phosphorylation by the upstream kinase (39). Thr172 on the α subunit is the main phosphorylation site of AMPK which leads to its activation (40), and has an important role in metabolic stress and in aging process (41). There are different kinases that activates the AMPK from Thr172 site (42). First, liver kinase L1 (LKB1) was found one of the activators of the AMPK and later on a study on HeLa cells showed that calcium/calmodulin-dependent protein kinase (CaMKKβ) could also activate the AMPK from Thr172 (43,44).

2.3.2. AMPK in macrophages

AMPK has a role in immunity. AMPK acts as a regulator of inflammatory pathways in macrophages. The role of AMPK in inflammation was demonstrated for the first time by Sağ et al. This study showed that AMPK plays an important role in suppression of inflammation and provides M2 macrophage polarization. Another study has showed that when bone marrow derived macrophages were isolated from AMPK -/- mice and stimulated with LPS, they showed increased TNFα and IL-6 production and decreased IL-10 production compared to WT mice (38). And different studies have shown that

this anti-inflammatory feature of AMPK is related with inhibition of NFkB signaling pathway(45).

One of the first pharmacologically proven activator of AMPK is 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) (46,47) . AICAR is an adenosine analog, converted to ZMP that mimics AMP in the cell. Similar to AMP, ZMP binds to the AMPKγ subunit, causing its allosteric activation (48). Studies with RAW264.7 macrophages showed that when these macrophages were pre-treated with AICAR, they showed decreased TNFα expression after LPS stimulation (49). LPS stimulated human macrophages showed increased AMPK activation and decreased TNFα and IL-6 expression after treatment with AICAR (47). LPS can induce activation of NF-kB by phosphorylating its upstream elements (50). NF-kB activation promotes production of pro-inflammatory cytokines. When murine macrophage RAW264.7 cell line was stimulated with LPS, it showed increased NF-kB phosphorylation, and when AMPK activator (ENERGI-F704) was used, it decreased the phosphorylation of NF-kB and decreased the production of pro-inflammatory cytokines such as IL-6, IL-8 and TNFα(51).

2.3.3. Relationship of AMPK and ABCG1

The connection between AMPK and ABCG1 has been shown in different studies. It has been shown that, human aortic endothelial cells stimulated with AICAR (AMPK activator) showed increased ABCG1 expression (52). AICAR stimulation led to increased ABCG1 expression also in murine macrophage cell line (J774.A1) (53). Another study reported that, macrophage foam cells incubated with AICAR showed increased cholesterol efflux compared to WT macrophages. Also, if AMPK in macrophage foam cells was inhibited, the cholesterol efflux was decreased (53). These studies reveals a correlation between AMPK and ABCG1.

2.4. Akt Protein

2.4.1. Akt Protein in Signaling Cascade

Akt (also known as Protein Kinase B- PKB) is a serine-threonine kinase that plays a role in cell metabolism, survival and proliferation (54). There are three isoforms of Akt protein in mice and humans which have 80% similarities in their amino acid sequence (55). Different isoforms of Akt protein are encoded by different genes and have different functions (56). For example, Akt1 is responsible for cell survival and proliferation; while Akt2 is responsible for glucose homeostasis and Akt3 is responsible for brain development (56,57). Activation of different isoforms occurs via phosphorylation from different sites: such as Akt1 via Ser473 and Thr308 and, Akt2 via Ser474 and Thr309 and Akt3 via Ser472 and Thr305, respectively (58).

PIP3 and PI(3,4)P2 metabolites are produced by PI3K which help to activate Akt via phosphorylation of Ser473 (59) by the mTOR complex 2 (mTORC2) (60). This phosphorylation is necessary for PI3K localization to cell membrane and to maintain the phosphorylation of Ser473 (59). Also, phosphorylation of (phosphoinositide-dependent kinase-1) PDK1 by PIP3 regulates the phosphorylation of Akt from Thr308 site (60).

One of the downstream targets of Akt is the Mammalian Target of Rapamycin (mTOR). Akt/mTOR pathway has several roles in the cell. They regulate protein synthesis through mTORC1 and they also play a role in cell survival (61). Akt also promotes cell survival via phosphorylation/inhibition of GSK3b (62).

2.4.2. Akt Signaling Pathway in Macrophages

Different Akt isoforms have different roles in macrophage polarization. It has been reported that peritoneal macrophages from Akt1-/- mice showed increased levels of pro-inflammatory cytokines, while macrophages from Akt2-/- mice showed a decrease

in pro-inflammatory cytokine production (63). In other words, Akt1 promotes M2 polarization, while Akt2 provides M1 polarization (64).

Previous studies have shown that PI3K/Akt pathway supports anti-inflammatory pathway and suppresses inflammation in macrophages (65). Pi3kr1 is a gene that encodes 3 different subunits of PI3K. It has been found that Pi3kr1 deficient mice peritoneal macrophages showed negative regulation between PI3K/Akt pathway and LPS stimulation (66). Akt is an important protein in PI3K/Akt signaling which provides an alternative activation by IL-4 stimulation in mice (67).

2.4.3. Relatioship of AMPK and Akt

In has been shown that AMPK-/- bone marrow derived macrophages displayed decreased phosphorylation of Akt from both sites (Thr308 and Ser473) (38). Also, dominant negative AMPK (DN-AMPK) B6J2 macrophage cell line showed decreased Akt phosphorylation after stimulation with LPS. In contrast, constitutively active AMPK (CA- AMPK) macrophages displayed enhanced Akt activation/phosphorylation after LPS stimulation (48). In another study, when WT bone marrow derived macrophages (BMDMs) were stimulated with IL-10, they showed increased Akt phosphorylation on both Thr308 and Ser473 and in AMPK -/- macrophages phosphorylation of Akt was decreased (68).

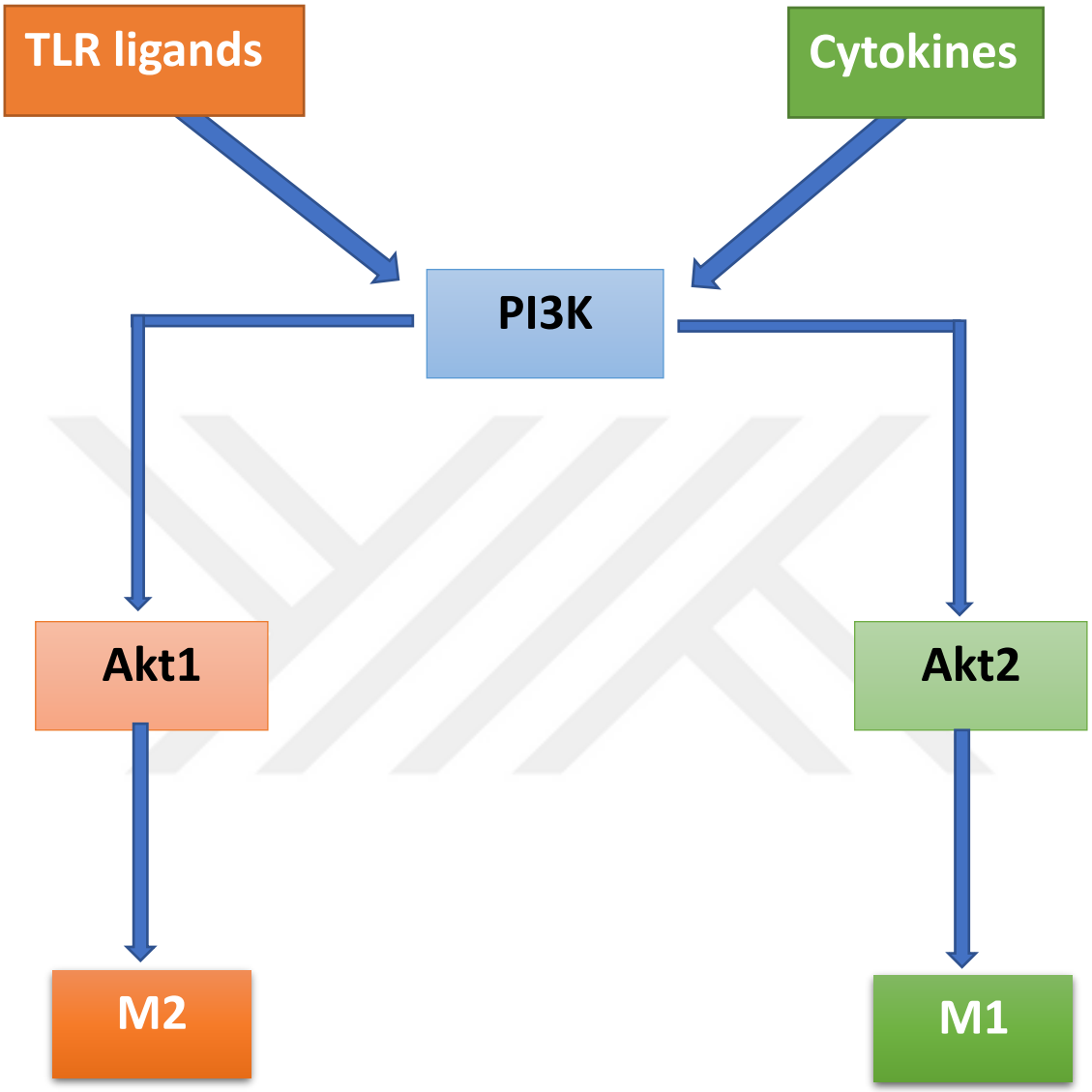


Figure 4. Akt isoforms in M1 and M2 macrophages. Activation of different isoforms of Akt in M1 and M2 macrophages.

3. MATERIALS AND METHODS

3.1. Type of the Study

The type of the study is experimental

3.2. Time and Location of the Study

Experimental procedures were carried out at Sağ Lab at İzmir Biomedicine and Genome Center between January 2018- May 2019.

3.3. The Universe and Sample of Research

Human Primary samples weren't used in this study.

3.4. Working Materials

We used bone marrow of WT (B6) and ABCG1 -/- mice for our experiments.

3.4.1. Reagents

Western Blot antibodies were purchased from Cell Signaling Technologies, name and catalog number of the antibodies as follow; PhosphoPlus® Akt (Ser473) Antibody Duet (Cat No: 8200S), PhosphoPlus® AMPK (Thr172) Antibody Duet (Cat No: 8208), Anti-rabbit IgG HRP-linked Antibody (Cat No: 7074). Color protein standard was purchased from NEB (Cat No: P7712S), BCA assay kit was purchased from Thermo (Cat No: 23227), Halt Protease Inhibitor Single-Use Cocktail was purchased from Thermo (78425), Halt Phosphatase Inhibitor Single-Use Cocktail was purchased from Thermo (Cat No: 78428), RIPA Buffer was purchased from Thermo (Cat No: 89901), ELISA KIT was purchased from eBioscience.

3.4.2. Chemicals that are used in Western Blot

3.4.2.1. Strip buffer

For 100ml Strip buffer,

1. 60ml distilled water was added to a glass .
2. 0.76g Tris base, 2g SDS and 700ul b-mercaptoethanol were added to the water.
3. pH was adjusted to 6.7.
4. Distilled water was added untill 100ml final volume.

3.4.2.2. For stripping procedure;

1. Water bath was adjusted to 55 °C.
2. 50 ml falcon tubes were filled up to 25ml with strip buffer and membrane pieces were put into tubes.
3. Then they were put in the water bath for 30 minutes as horizontal.
4. After every 10 mins tubes were taken from water bath and shaken by hand and put into water bath again.
5. Rest of the procedure was same as blotting part of western blot.

3.4.2.3. 8% Resolving Gel (For 20ml):

1. 9.3 ml distilled water.
2. 5.3 ml 30% acryl-bisacrilamide mix
3. 5.0 ml 1.5 ml Tris (pH 8.8)
4. 200ul 10% SDS
5. 200ul 10% amonium persulfate
6. 12ul TEMED

3.4.2.4. 5% Stacking Gel (For 6 ml):

1. 4.1 ml distilled water

2. 1 ml 30% acryl-bisacrylamide mix
3. 750ul 0.5M Tris (pH 6.8)
4. 60ul 10% SDS
5. 60ul 10% amonium persulfate
6. 6ul TEMED

3.4.2.5. 10X Running Buffer

1. 10g SDS
2. 30.3g Tris
3. 144.1 g Glycine

The chemicals above were first mixed in 800 ml distilled water and after they were dissolved, 200 ml distilled water was added for final volume.

3.4.2.6. Preparation of 1.5 M Tris pH 8.8

1. 18.15g Tris was dissolved in 90 ml distilled water.
2. pH was adjusted to 8.8 by adding HCl.
3. Distilled water was added to make the final volume 100ml

3.4.2.7. Preparation of 0.5 M Tris pH 6.8

1. 6g Tris was dissolved in 90 ml distilled water
2. pH was adjusted to 6.8 by adding HCl
3. Distilled water was added to make the final volume 100ml

3.5. Study Variables

Variables of our study are LPS, IFNγ, IL-4 and the Akt Activator (SC79)

3.6. Data Collection Tools

3.6.1. Generation of Bone Marrow-Derived Macrophages (BMDMs)

Femur and tibia of ABCG1$^{-/-}$ and B6 (WT control) mice were taken and put into drilled 0.6ul pcr tubes. Tubes were taken into 1.5ml eppendorf tubes and centrifugated for 7000 RPM for 15 secs. After centrifugation, bone marrow cells were in 1.5ml eppendorf tubes. The cells were resuspended in R5 medium -which contains 5% heat-inactivated FBS (Gibco, Cat. No: 10500-064), 1% penicillin/Streptomycin, 1% L-Glutamine. 10ng/ml M-CSF(PeproTech, cat no: 315-02) were added to the cells. After this step, cells were plated in a 100 mm culture dish and incubated at 37°C. The next day, the non-adherent cells were transferred to a 50 ml falcon tube. L929 conditioned medium from L929 fibroblast cell line (which produces M-CSF) was added as 30% on the cells. The mix was made as follows;

10 ml bone marrow cells (non-adherent cells) from the initial culture

7.5 ml R5 medium

7.5 ml L929 condition medium

10 ng/ml M-CSF

25 ml total volume

The mix was transferred to an ultra-low attachment 6 well plates (Corning) as 4 ml/well then incubated at 37°C for 7 days. At day 3 and 5, 25ml mixed medium prepared with 12.5ml R5 medium + 12.5ml L929 conditioned medium + 10ng/ml M-CSF was added on both ABCG1$^{-/-}$ and WT cells as 2 ml for each well. On day 7, cells were taken with vigorous pipetting and washed with 1X PBS once at 300 RCF for 5 minutes. Then, cells were resuspended in 5ml R5 medium and layed onto 2ml Ficoll in 15ml falcon tube (Ficoll should be preserved at dark). Both tubes (ABCG1-/- and WT cells) were centrifuged (Eppendorf, Centrifuge 5810R) at 300 RCF w/o breaks –

gradient centrifugation- for 20 minutes. The cells that were seen in the middle interphase -macrophages- were collected and washed once with 1X PBS at 300 RCF for 5 minutes. Then, cells were resuspended with 5ml R5 medium in 15ml falcon tube and counted on hemocytometer. Lastly, cells were plated on a 24 well plate as $1x10^6$ cells/well in 1 ml R5 medium for Western blot or on a 96 well plate as $1x\ 10^5$ells/well for ELISA analyses. Plates were incubated at 37°C for 1 day for resting. The following day, BMDMs were stimulated with LPS (100ng/ml)(InvivoGen, Cat. No: tlrl-3pelps)+ IFN-γ (20 ng/ml)(R&D, Cat. No: 485-MI) for M1 polarization and with IL-4 (20 ng/ml)(R&D, Cat. No: 404-ML) for M2 polarization at different time points.

3.7. Protein Concentration Measurement

BMDMs were stimulated with M1 and M2 stimulants for 10, 30, 60, 120 mins, 6 hrs or 18 hrs. The cells were taken on ice immediately and the medium replaced with fresh R5 medium that doesn't contain stimulants. After that, the cells in each well were washed with 1X PBS (Gibco, Cat. No: 10010-015) twice and then lysed with 100ul RIPA buffer that contains Halt protease and phosphatase inhibitor cocktail for 5 mins on ice. Lysates were taken into 1.5ml eppendorf tubes and vortexed for 30 secs and centrifuged (Thermo, MicroCL 17R) at 14.000 RCF for 15 mins at 4 ^{0}C. Supernatants were collected into new tubes and stored at -80^{0}C if they were not used immediately.

Protein concentration of lysates was measured by BCA assay kit. Standards of BCA assay were prepared as recommended in the assay protocol. Our samples were diluted 1/12 with distilled water and prepared in 0.6ul tubes. Before they were added into the each well of a 96 well cell culture plate, every sample was vortexed for 5 secs and added as 25ul to each well. After that, working reagent, which was prepared according to the number of samples (we prepared the samples duplicate) from A and B solution as recommended in the protocol, was added as 200ul onto each well, and wrapped with aluminum foil, and kept in the incubator at 37^{0}C for 30 mins. Then the samples were analyzed with Multiscan Go at 560nm. The results were calculated with GraphPad

prism software, and an equal amount of protein from each sample was prepared for Western blot.

3.7.1. Western Blot Analysis

For the Western blot analysis, firstly, the Polyacrylamide gel was prepared and kept at room temperature (RT) for 2 hours (when needed to be used the next day, after 2 hrs of RT, it was stored at +4 ^{0}C overnight). The samples were prepared and 30ug protein was used from each sample. Laemmli buffer was prepared as 2X and stored at -20 ^{0}C w/o adding any β-mercaptoethanol (Applichem, Cat. No: A4338). Before the usage of laemmli buffer, 50ul β-mercaptoethanol was added into the tube and mixed gently. The samples and the laemmli buffer was mixed as 1:2 and kept on ice. After this step, samples were put on in 95^{0}C heat block for 5 minutes and then taken to be loaded to the gel.

Proteins were run in the at 60 V first and after they passed through the resolving gel, voltage was increased to 100. We used 5% stacking and 8% separating gel. After the running part, for the transfer of the proteins to the membrane transfer, buffer was prepared fresh with 1 lt distilled water, 3.75gr glycine(Applichem, Cat. No: A1067) and 7.25 trisma base(Sigma, Cat. No: T6066) and lastly, before the preparation of the sandwich with membrane and gel, 250ml methanol was added. After that, PVDF membrane (Millipore, Immobilon-FL Cat. No: IPFL00010) was cut like exactly the same size with gels. Then they were put into methanol for 5 minutes to be activated. In this 5 min. period the sandwich was prepared as follows;

1. First the cassette was opened and the black part was hold at the bottom
2. The black sponge was wet with the transfer buffer and put on the black part of the cassette.
3. Blot papers were wet with transfer buffer and put onto the sponge (we usually use 2 blot papers for one side and two for other side).
4. The gel was taken gently and added on the papers.

5. After 5 minutes activation of PVDF membrane, it was taken and put onto the gel.
6. The 2nd and the 3rd steps were repeated respectively.
7. Prepared sandwich was put into the transfer tank and filled with transfer buffer up to the 2 gels line.
8. The thank was put into the small container and around the tank was filled with ice and taken to the cold room for over-night incubation (17-18 hours).

The following day, the membranes were taken and put into methanol for 1 minute (to fix the proteins onto the membrane for another usage of the membranes in strip procedure) and then blocked with 1% BSA(Applichem, Cat. No: A6588) in TBS-T for 2 hours. After the blocking, membranes were incubated with primary antibodies (1/2000 ratio for each antibody in 1%BSA) for 1 hour. Then, they were washed twice and each time for 30 mins in TBS-T (1X TBS + 1 ml Tween (for 1L)). Membranes were incubated with secondary antibody (1/2000 ratio for secondary antibody in 1% BSA) for 1 hour after the washing part. At the end of the 1 hour incubation, the membranes were washed again as described before and the proteins were detected by using ECL (Millipore, Cat. No: WBLUF0500) system. Densitometry analysis was done by using the ImageJ program.

3.7.2. ELISA

BMDMs were plated in a 96-well cell culture plate as $1x10^5$ cells in 200 ul R5 medium for each well and rested for 1 day at 37 ^{0}C to be used for ELISA. The next day, they were pre-treated with Akt Activator (8ug/ml)(Sellekchem, Cat No: S7863) for 2 hours and afterwards stimulated with LPS (100ng/ml)+IFN-γ (20ng/ml) for M1 polarization overnight. After this period, supernatants were collected and stored at -20 ^{0}C. We used 10 ul supernatant and added 90ul ELISA dilution buffer to dilute it 1/10. TNF-α production was measured according to the protocol of the manufacturer.

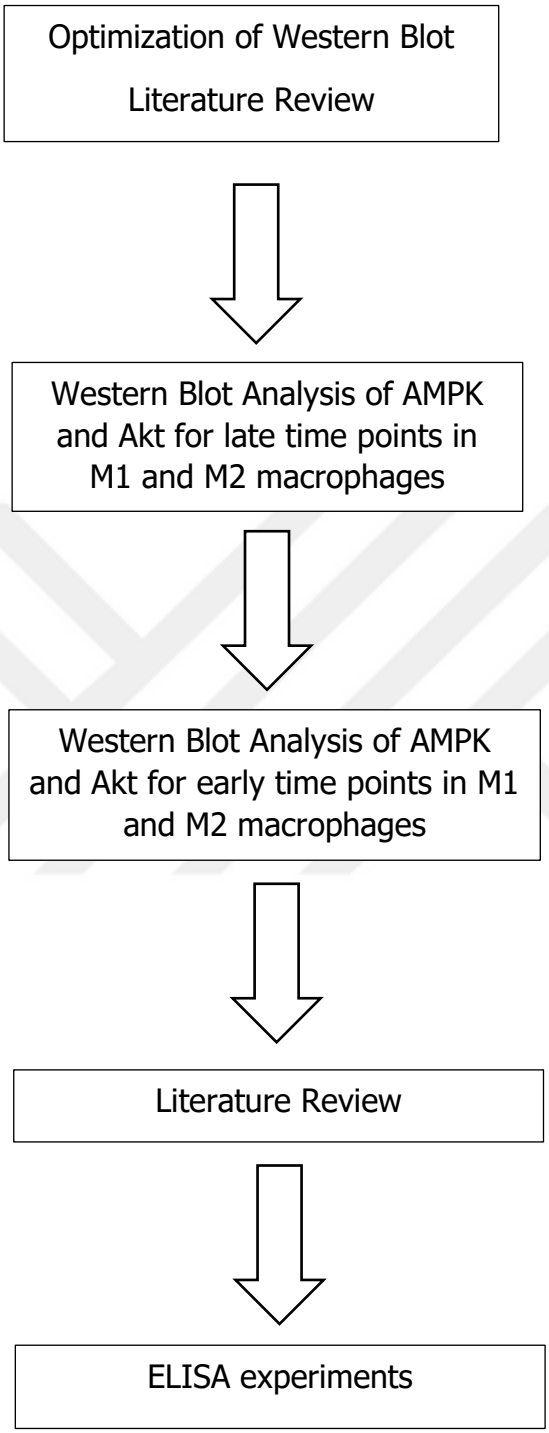
1. 10X coating buffer was diluted with distilled water to 1X and added to each well as 100 ul. Then it was preserved at 4 ^{0}C overnight (18 hours).
2. After 18 hours, the plate was taken from 4 ^{0}C and washed 3 times with washing buffer. Washing buffer contained 1X PBS and 0,05% Tween.
3. 5X ELISA/ELISPOT diluent was diluted to 1X with distilled water and used as 200 ul for each well for blocking. We blocked the samples for 1 hour on shaker at room temperature.
4. After 1 hour blocking, blocking buffer was removed and washed one time with washing buffer and dried until all buffer droplets were removed.
5. After the washing step, the standards were prepared. 1 ml distilled water was added to the recombinant TNF-α vial. And then, the standards were prepared as duplicates as recommended by the manufacturer's protocol.
6. The samples were prepared as 10 ul sample+ 90 ul ELISA diluent and incubated for 2 hours on shaker at room temperature.
7. After incubation period, the plate was washed with washing buffer for 4 times and 1 time with distilled water.
8. The detection antibody was prepared in 1X ELISA diluent as recommended in the protocol. 48 ul antibody was added to 12 ml ELISA diluent for 96 well cell culture plate. 100 ul was added into each well and incubated at room temperature on the shaker for one hour.
9. After this step, the plate was washed 4 times with washing buffer and once with distilled water and then dried.
10. The HRP conjugated antibody was prepared the same as detection antibody and incubated for 30 mins in the dark at room temperature.
11. After this step, the plate was washed 6 times by washing buffer and once with distilled water and then dried.
12. Lastly, 100 ul TMB solution was added into each well and incubated for 10 mins in the dark. Then 50 ul stop solution was added into each well to stop the reaction. And the samples were analyzed by the multi-scan reader at 450nm.

3.7.3. Flow Cytometry Analysis

WT and ABCG1 -/- mice bone marrow derived macrophages were plated in 48 well plate as $35x10^4$ in each well and incubated at 37 C for a day. The next day, the cells were prepared for cell surface staining as follows;

1. All steps were done on ice until cell fixation (staining of alive cells requires cold temperature)
2. The cells were resuspended with 1 ml FACS Buffer (0.25% Na-azide /1%BSA in 1X PBS) and transferred into 1.5 ml tubes and centrifuged at 400 Rcf for 5 min. (Washing step).
3. After aspiration, each tube was resuspended with 50 ul Fc block (dilution 1/200 in FACS buffer) and the cells were incubated for 10 mins on ice.
4. Cell surface staining antibodies were prepared in FACS buffer at 1/200 dilution then directly added as 50ul to each tube containing Fc block.
5. The cells were incubated in dark on ice for 30-45 minutes.
6. FACS buffer was added as 1ml to each tube to stop the staining process and the tubes were centrifugated at 4^0C. This step is repeated 1 more time. (Washing step)
7. 100 ul Cytofix-Fixation Medium was added to each tube and incubated for 15-20 minutes in the dark on ice.
8. 1 ml FACS buffer was added to each tube to stop the fixation process and the tubes were centrifuged at 400 Rcf for 5 min. (after this fixation step, the cells could be centrifuged at RT).
9. The cells were washed once more with 1 ml FACS buffer.
10. The cells were resuspended with 100 ul FACS buffer and preserved at 4 ^{0}C in the dark until analysis.
11. It was analysed by FlowJo.

3.8. Study Plan



3.9. Evaluation of Data

Statistical Analysis: Student's t test was used for comparison of experimental groups. P values of less than 0.05 were considered statistically significant.

3.10. Limitations of the Study

There were no limitations of our study.

4. RESULTS

4.1. Macrophages (%)

To study the impact of ABCG1 deficiency on macrophages, WT and Abcg1$^{-/-}$ bone marrow-derived macrophages (BMDMs) were generated from WT and Abcg1$^{-/-}$ mice. To verify that the cells were indeed macrophages, we analyzed the expression of mouse pan-macrophage markers F4/80 and CD11b by flow cytometry. We found that 90.72% of WT and 93.13% of ABCG1 -/- bone marrow derived cells were F4/80+, CD11b+ macrophages (Figure 5).

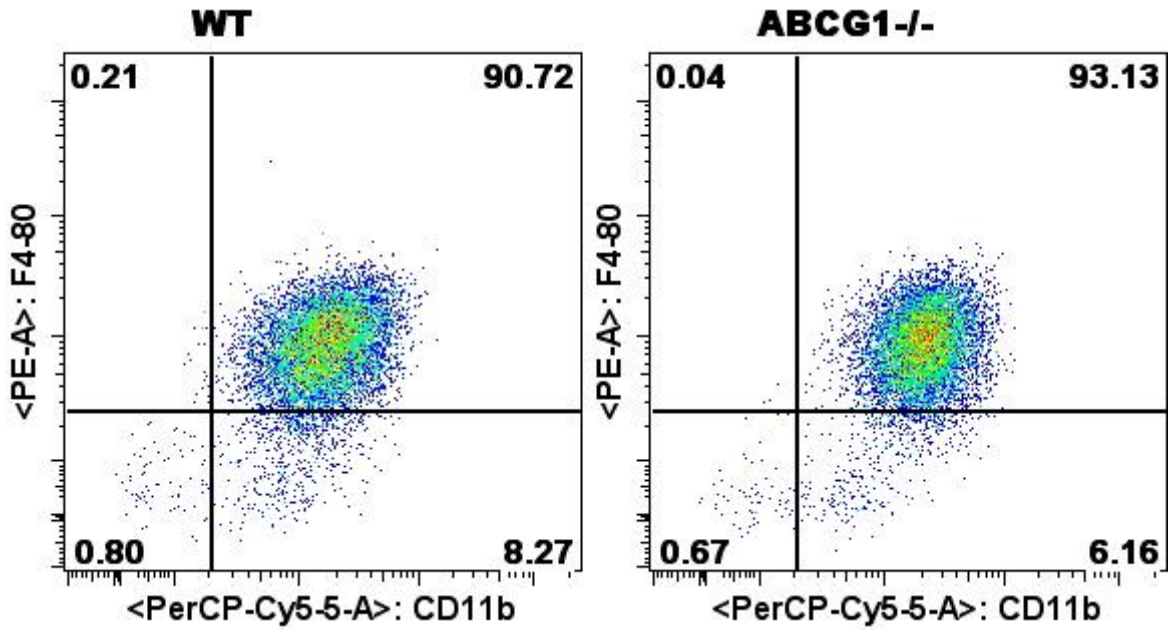


Figure 5. Percentages of WT and ABCG1 -/- bone marrow derived macrophages. Expression of macrophage markers such as F4/80 and CD11b were analysed in Abcg1-/- and WT bone marrow derived macrophages by flow cytometry. The data was analyzed by FlowJo.

4.2. AMPK activation in WT and ABCG1 -/- Macrophages

Previously, we have shown that Abcg1 -/- macrophages intrinsically shift towards a tumor-fighting M1 phenotype and suppress tumor growth in mice. This shift is associated with increased NF-kB activation (48). However, the molecular mechanism of this shift is not known. Our main goal in this study was to uncover the underlying mechanism of this macrophage shift in Abcg1-/- macrophages.

AMPK is the master regulator of cellular energy homeostasis and metabolic stress. We and others have previously reported that AMPK plays a role in macrophage polarization (38,48,68). Activation of AMPK suppresses proinflammatory responses and promotes macrophage polarization to an M2 phenotype, whereas, inhibition of AMPK switches the phenotype of macrophages to M1(48). Inhibition of AMPK exerts its pro-inflammatory effects in macrophages through activation of NF-κB (48).

Studies on cholesterol transport report a positive correlation between ABCG1 expression and AMPK activation (53,56). Therefore, we first hypothesize that the absence of ABCG1 causes inhibition of AMPK and thereby NF-κB activation, which polarizes macrophages to a tumor-fighting M1 phenotype.

To determine the impact of ABCG1 deficiency on AMPK signaling, WT and Abcg1-/- BMDMs were stimulated with M1 signal IFNγ (20 ng/ml) + LPS (100 ng/ml) or M2 signal IL-4 (20 ng/ml) for 0, 10', 30', 1h, 2h, 6h and 18h. Western blot was performed using antibodies against p-AMPK (Thr172), total AMPK and b-actin.

We first stimulated the BMDMs from WT and Abcg1-/- for 1h, 6h and 18h with LPS+IFNγ and checked AMPK phosphorylation/ activation. At basal level, p-AMPK level was higher in WT BMDMs compared to Abcg1-/- BMDMs. However, 1 h after M1 stimulation , p-AMPK level was lower in Abcg1-/- macrophages. Interestingly, at the 6 h and 18h time points, p-AMPK level of Abcg1-/- macrophages were higher than WT. (Figure 6). After this unexpected result, we wanted to check earlier time points and stimulated WT and Abcg1 macrophages with LPS/IFNγ for 10' , 30' , 60' and 120' time periods. We found that, 10' post-stimulation, AMPK activation level was higher in

Abcg1-/- macrophages compared to WT. At 30', Abcg1-/- macrophages had still higher p-AMPK levels; however, the increase was less prominent compared to 10' time point (Figure 6). At 60', p- AMPK level was lower in Abcg1-/, as observed previously (Figures 6 and 7). At 120' p-AMPK was lower in Abcg1-/- macrophages; however the decrease was less prominent compared to 60' time point.

Next, we stimulated WT and Abcg1-/- BMDMs for 1h, 6h and 18h with M2 stimulus IL-4 and checked AMPK phosphorylation/ activation. At basal level and at all time points, p-AMPK level of Abcg1-/- BMDMs were higher compared to WT (Figure 8). After that, we wanted to check earlier time points and stimulated WT and Abcg1-/- macrophages with IL-4 for 10' , 30' , 60' and 120'. We found that at 10' time point, p-AMPK levels of both groups were similar. Starting from 30', p-AMPK level of Abcg1-/- macrophages were lower compared to WT macrophages (Figure 9). These results are not in line with our hypothesis.

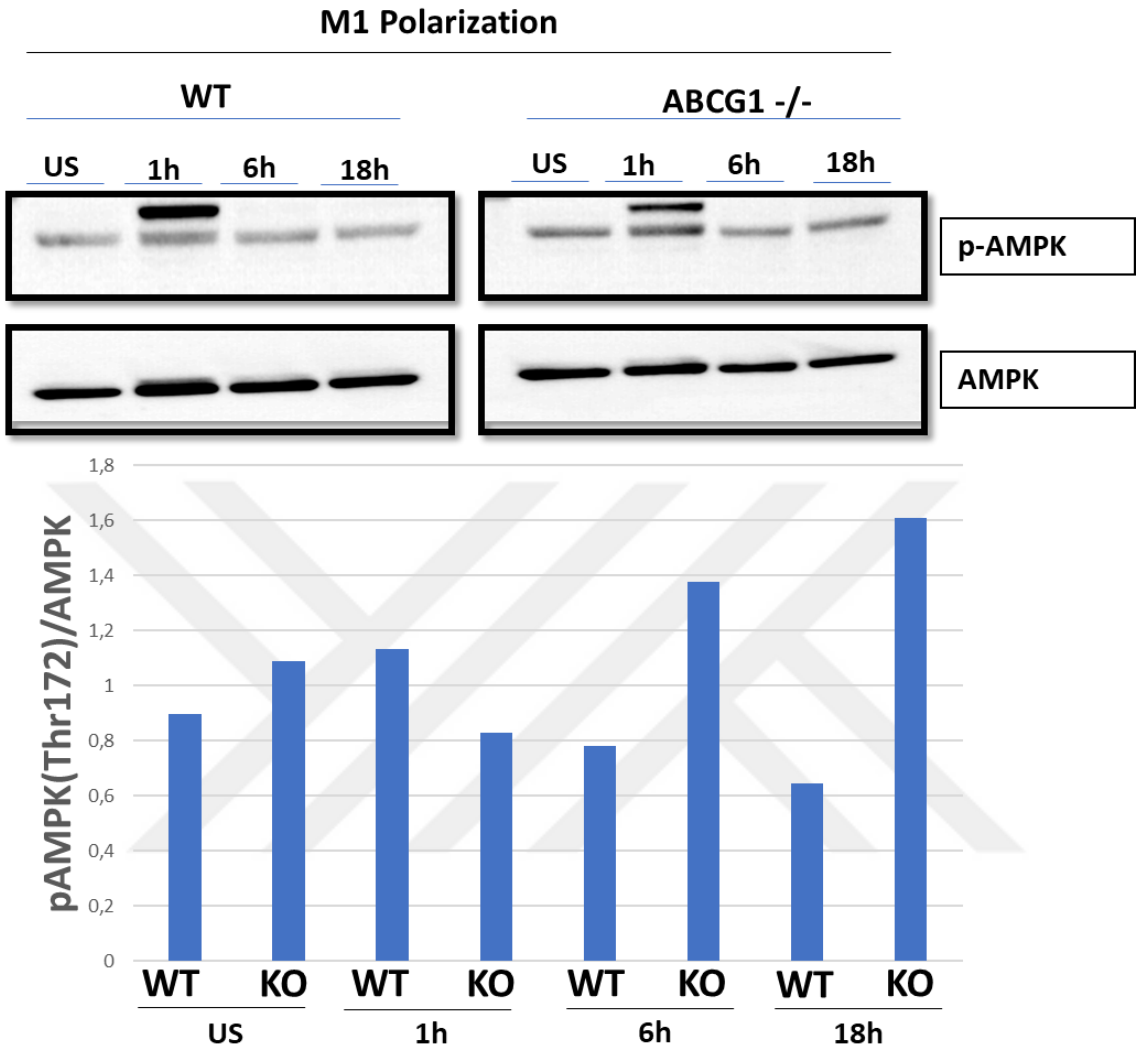


Figure 6. p-AMPK/AMPK ratio in WT and ABCG1 -/- M1 macrophages in late time points. WT and ABCG1 -/- BMDMs were stimulated with LPS+IFNγ for 1h, 6h and 18 h for M1 polarization. p-AMPK and total AMPK levels were analyzed by Western Blot. Blot bands were analyzed by Image J.

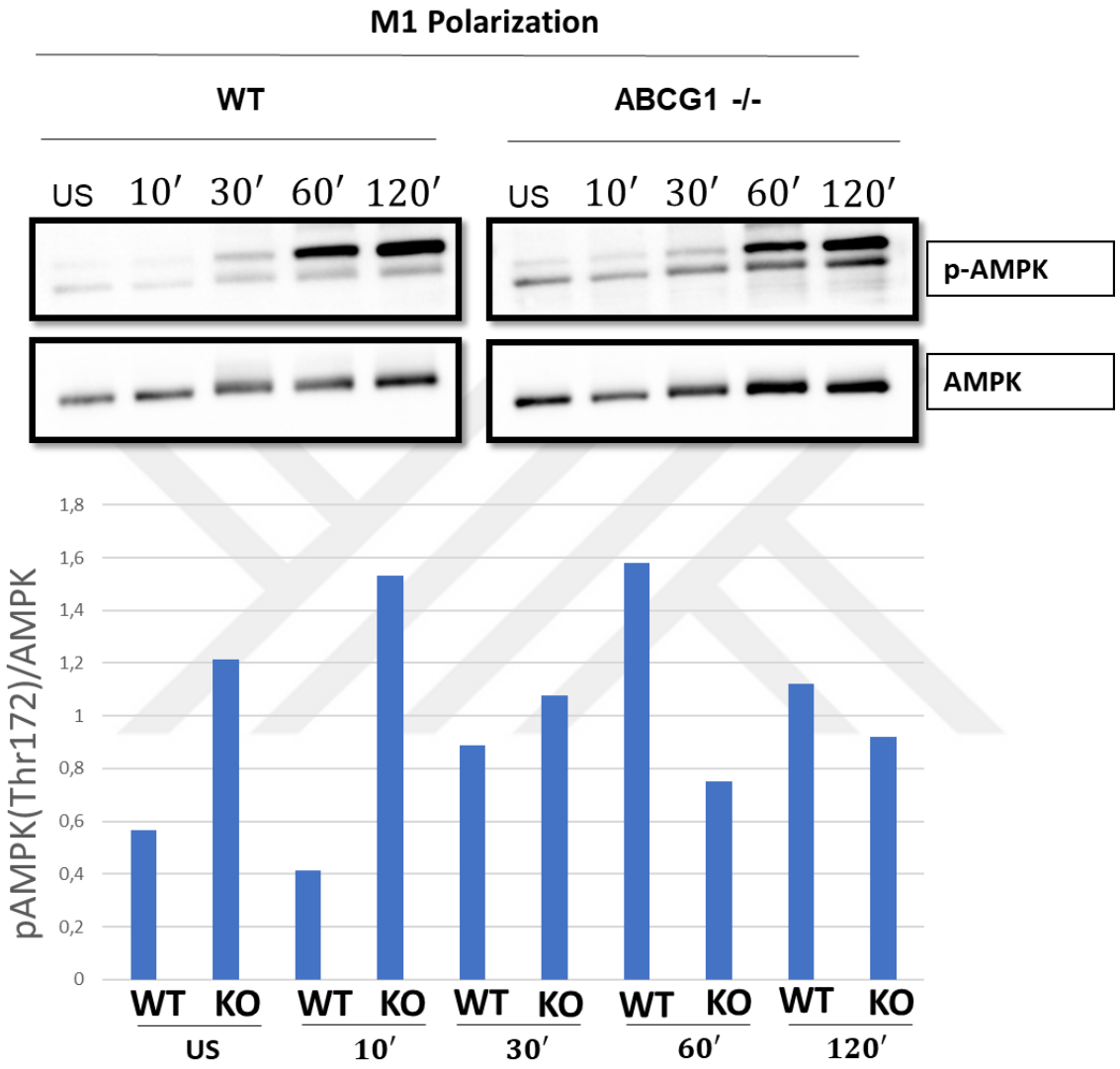


Figure 7. p-AMPK/AMPK ratio in WT and ABCG1 -/- M1 macrophages in early time points. WT and ABCG1 -/- BMDMs were stimulated with LPS+IFNγ for 10′, 30′ 60′ and 120′ for M1 polarization. p-AMPK and total AMPK levels were analyzed by Western Blot. Blot bands were analyzed by Image J.

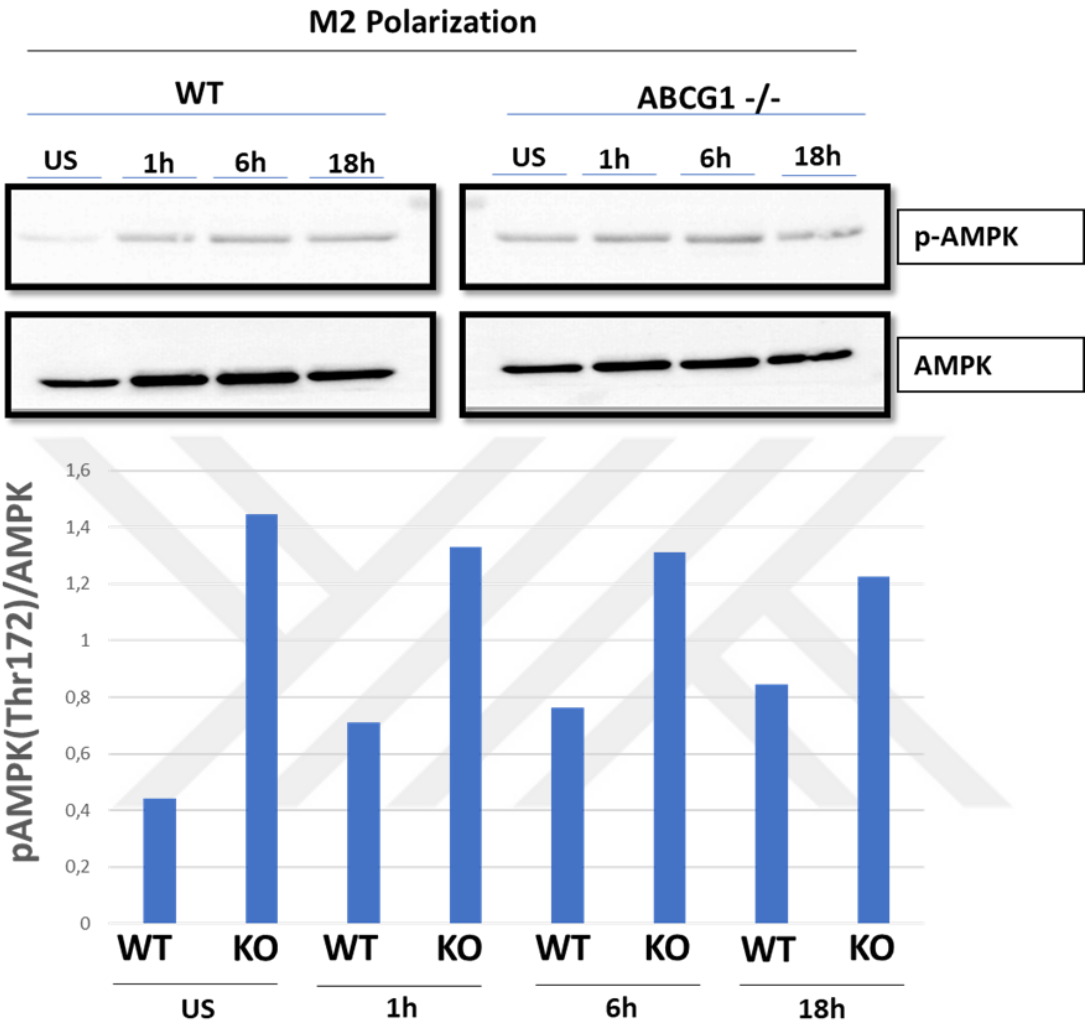


Figure 8. p-AMPK/AMPK ratio in WT and ABCG1 -/- M2 macrophages in late time points. WT and ABCG1 -/- BMDMs were stimulated with IL-4 for 1h, 6h and 18 h for M2 polarization. p-AMPK and total AMPK levels were analyzed by Western Blot. Blot bands were analyzed by Image J.

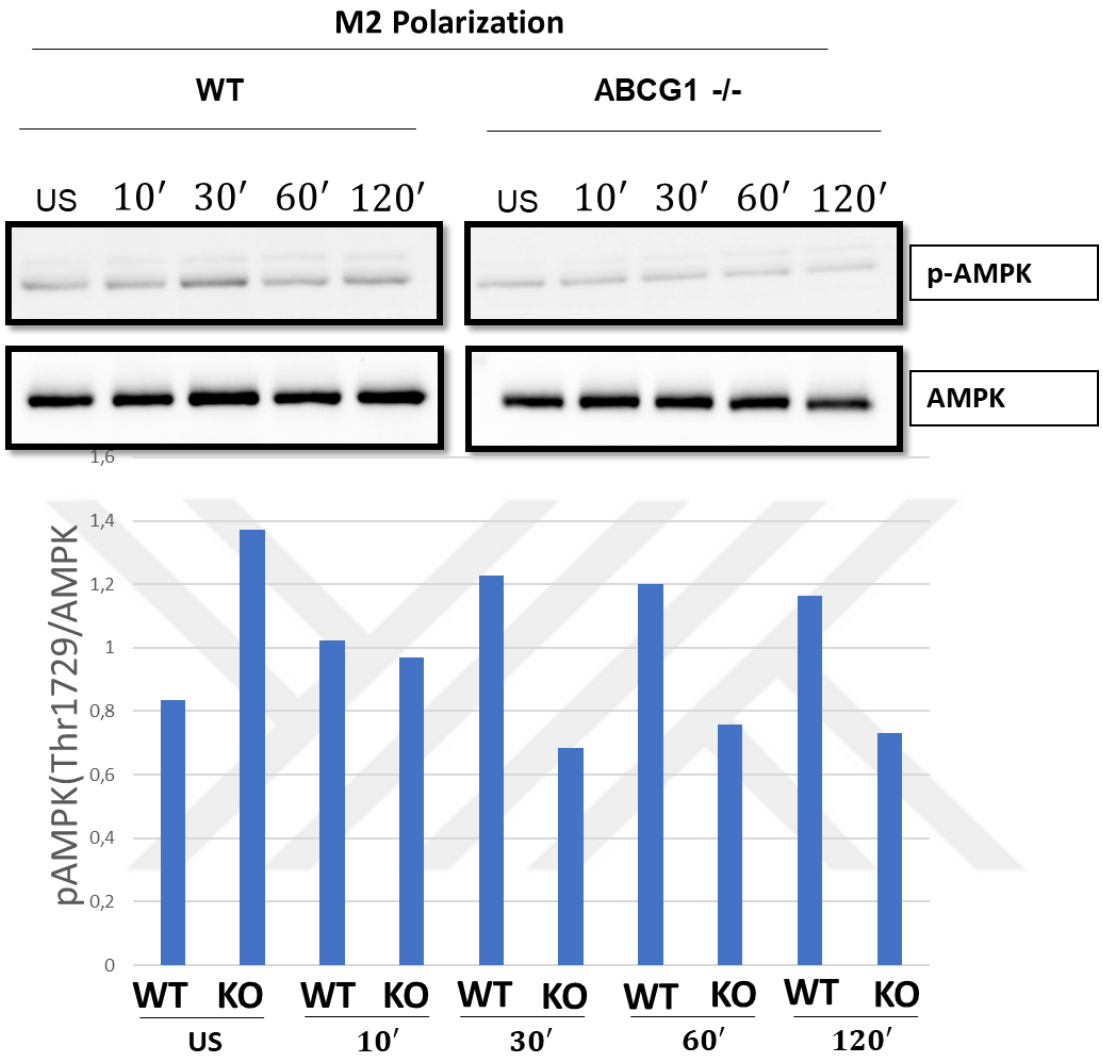


Figure 9. p-AMPK/AMPK ratio in WT and ABCG1 -/- M2 macrophages in early time points. WT and ABCG1 -/- BMDMs were stimulated with IL-4 for 10', 30' ,60' and 120' for M2 polarization. p-AMPK and total AMPK levels were analyzed by Western Blot. Blot bands were analyzed by Image J.

4.3. Akt1 activation in WT and ABCG1 -/- Macrophages

Previous studies showed that one of the downstream signaling molecules of AMPK in macrophages is Akt. Akt1 activation has been shown to promote M2 polarization of macrophages(63). Therefore, we hypothesize that the absence of ABCG1 causes inhibition of Akt1, which polarizes macrophages to a tumor-fighting M1 phenotype.

To test this hypothesis, Abcg1-/- and WT BMDMs were stimulated with IFNγ+LPS for M1 polarization for 10', 30', 1h, 2h, 6h and 18h. At basal level and all time points except 18 h, Abcg1-/- BMDMs displayed lower level of Akt1 activation compared to WT macrophages (Figure 10 and 11). At 18 h time point, Akt1 activation level of Abcg1-/- and WT macrophages was comparable (Figure 11).

Next, Abcg1-/- and WT BMDMs were stimulated with IL-4 for M2 polarization for 10', 30', 1h, 2h, 6h and 18h. Similar to the results of M1 stimulation, Abcg1-/- BMDMs exhibited lower p-Akt1 level compared to WT macrophages at basal level and all time points except 18 h (Figure 12 and 13). At 18 h time point, Akt1 activation level of Abcg1-/- and WT macrophages was again comparable (Figure 13).

These data suggest that Akt1 could be one of the signaling molecules in ABCG1-/- mice that play a role in the shift of macrophages towards M1 phenotype.

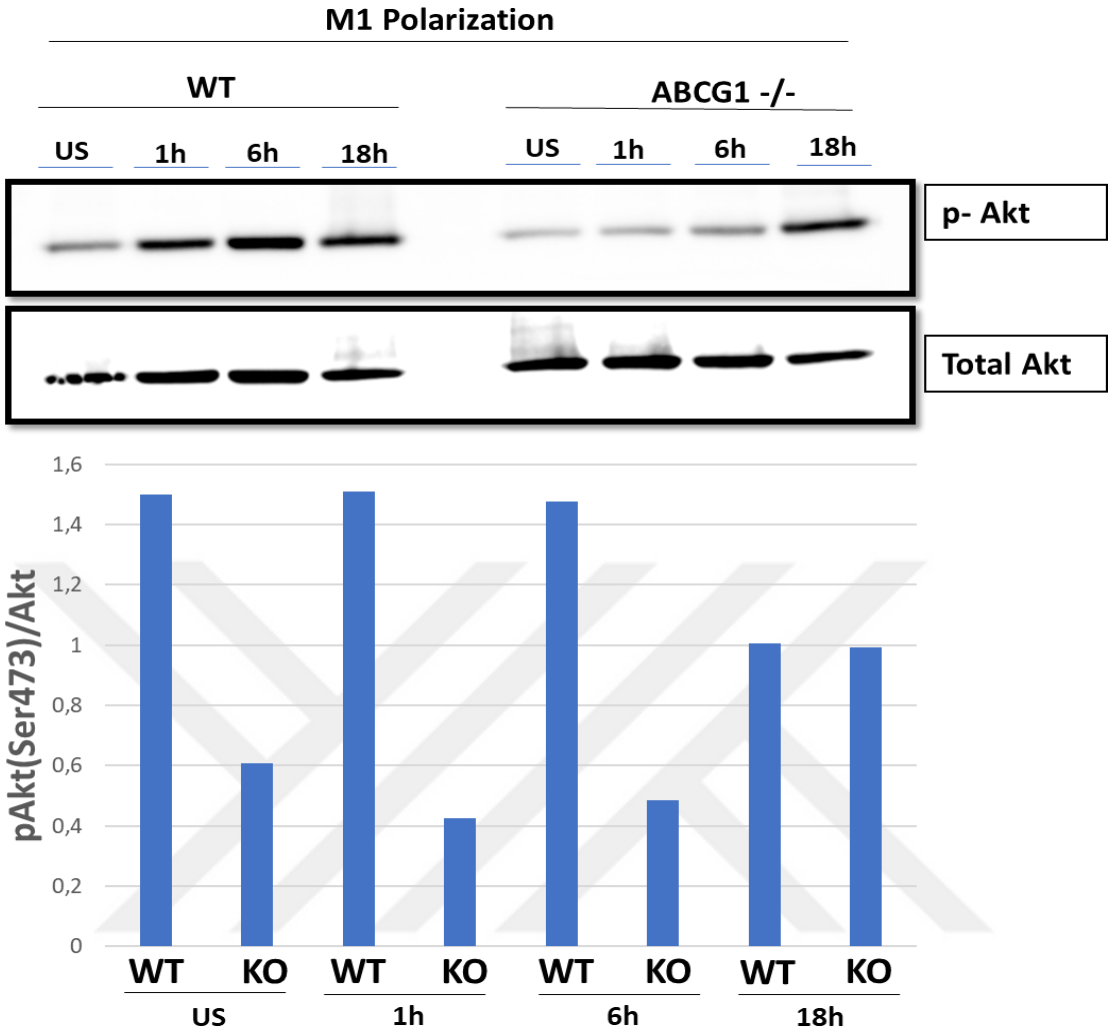


Figure 10. p-Akt/Akt ratio in WT and ABCG1 -/- M1 macrophages in late time points. WT and ABCG1 -/- BMDMs were stimulated with LPS+IFNγ for 1h, 6h and 18 h for M1 polarization. p-Akt1 and total Akt1 levels were analyzed by Western Blot. Blot bands were analyzed by Image J.

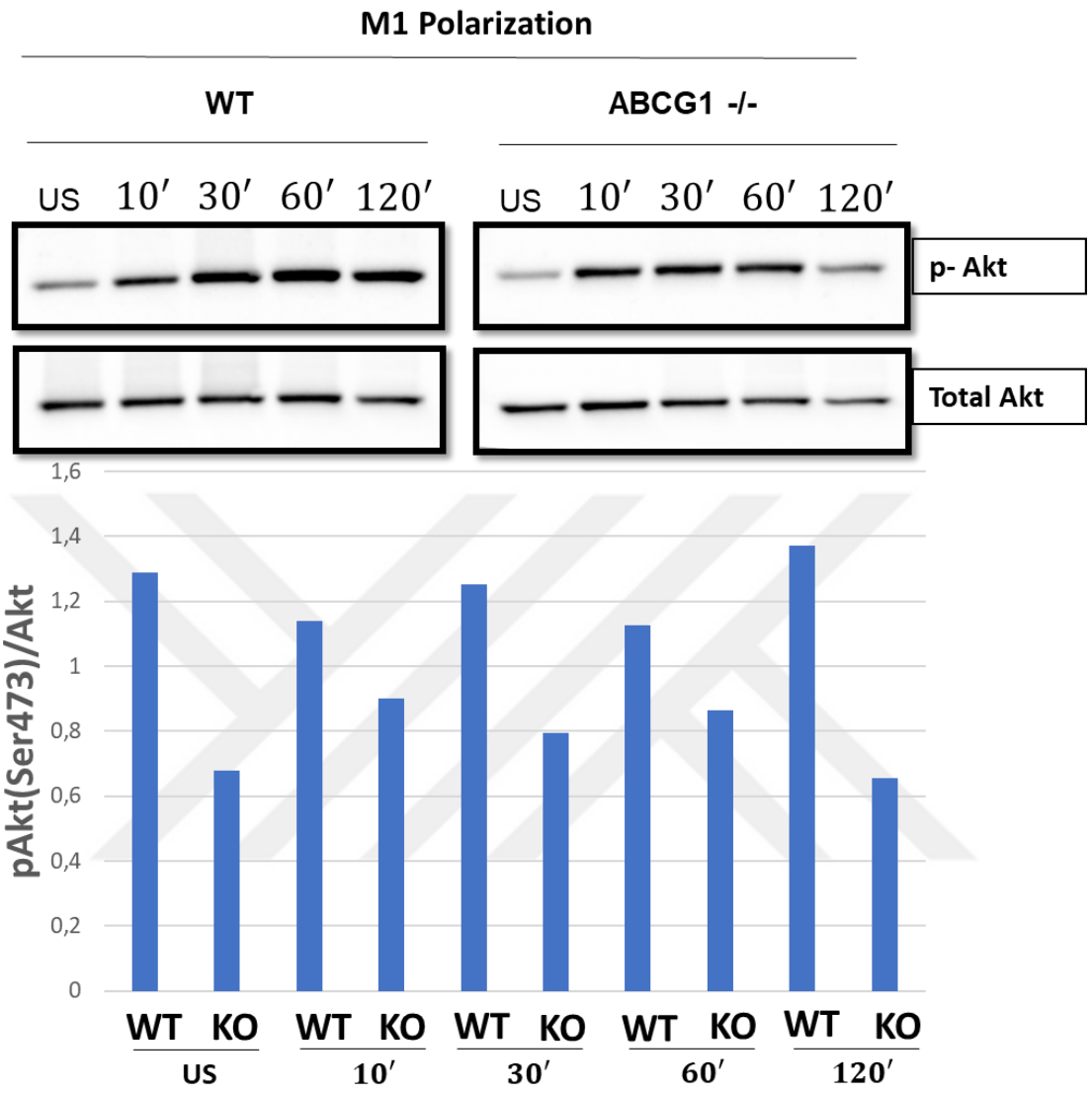


Figure 11. p-Akt/Akt ratio in WT and ABCG1 -/- M1 macrophages in early time points. WT and ABCG1 -/- BMDMs were stimulated with LPS+IFNγ for 10′, 30′ 60′ and 120′ for M1 polarization. p-Akt1 and total Akt1 levels were analyzed by Western Blot. Blot bands were analyzed by Image J.

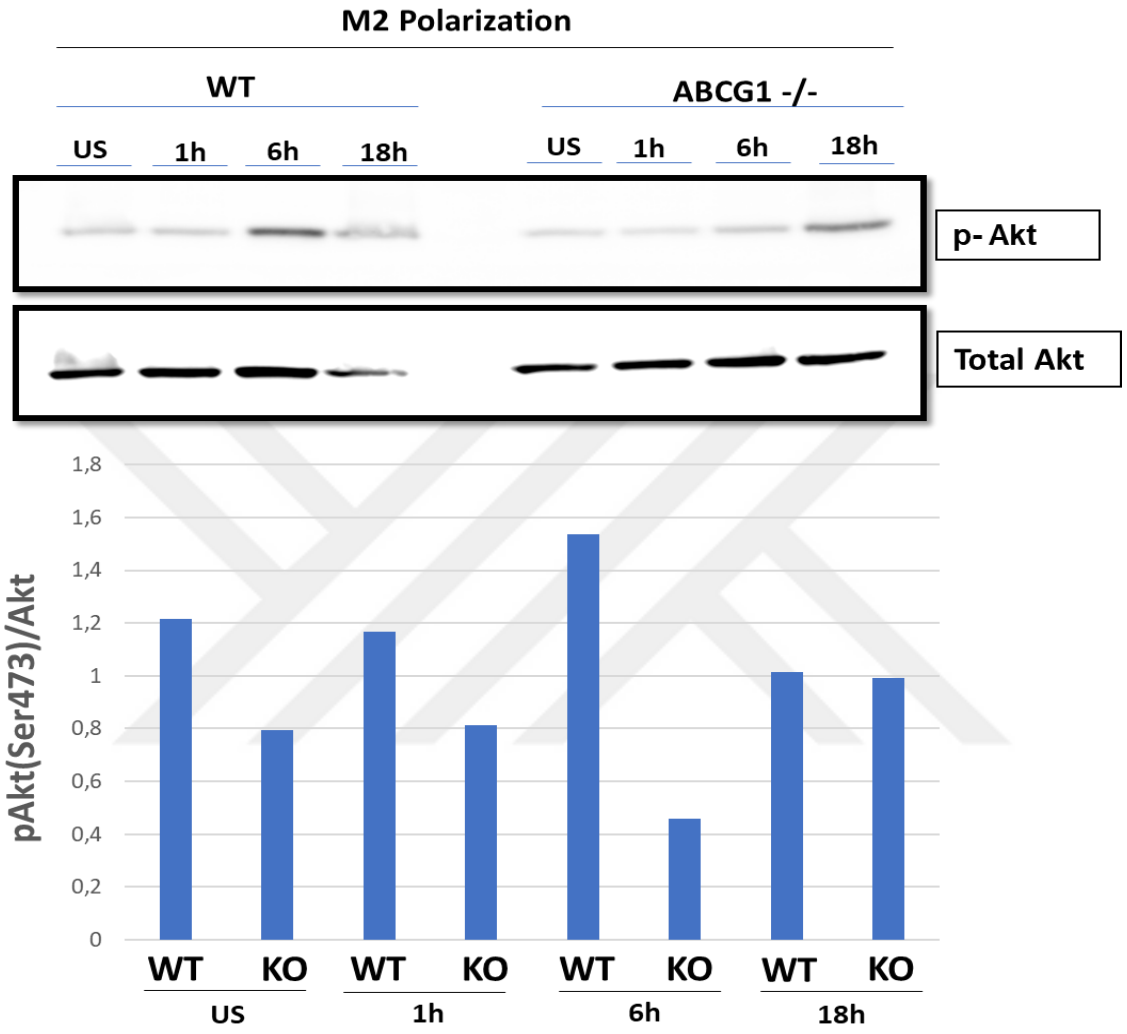


Figure 12. p-Akt/Akt ratio in WT and ABCG1 -/- M2 macrophages in late time points. WT and ABCG1 -/- BMDMs were stimulated with IL-4 for 1h, 6h and 18 h for M2 polarization. p-Akt1 and total Akt1 levels were analyzed by Western Blot. Blot bands were analyzed by Image J.

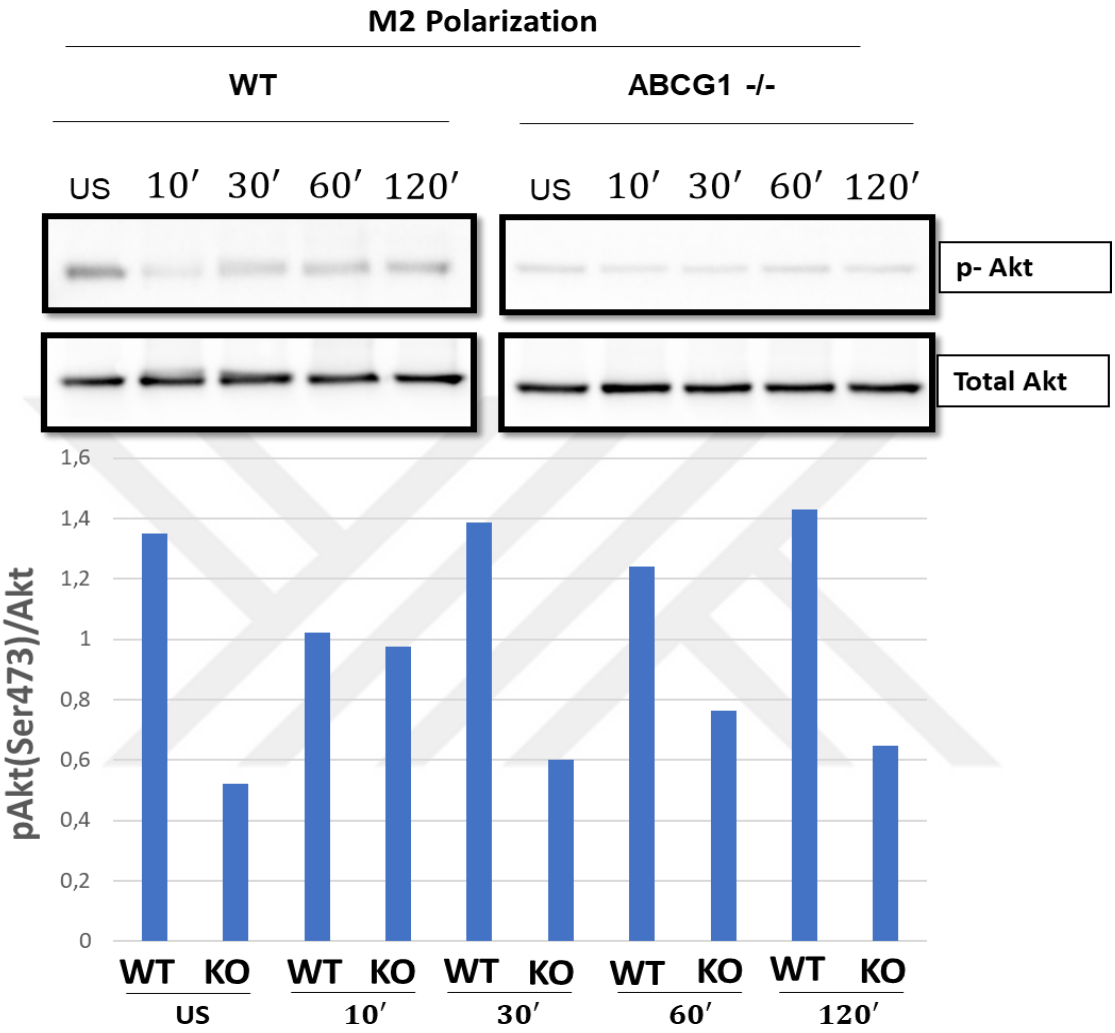


Figure 13. p-Akt/Akt ratio in WT and ABCG1 -/- M2 macrophages in early time points. WT and ABCG1 -/- BMDMs were stimulated with IL-4 for 10′, 30′ ,60′ and 120′ for M2 polarization. p-Akt1 and total Akt1 levels were analyzed by Western Blot. Blot bands were analyzed by Image J.

4.4. TNF-α production in Macrophages Treated with Akt Activator

As inhibition of Akt1 activation promotes macrophage polarization to an M1 phenotype (63), reduced Akt1 activation in Abcg1-/- macrophages would explain the observed M1 bias in these cells. If that was the case, then we would expect that the pre-treatment of macrophages with the Akt activator SC79 will decrease the production of TNFα, and revert the phenotype of Abcg1-/- macrophages to WT.

Hence, Abcg1-/- and WT BMDMs were pre-incubated with the pharmacological Akt activator SC79 (8ug/ml) for 2 hours or left untreated. After that, they were stimulated with LPS (100ng/ml)+IFNγ (20ng/ml) for M1 polarization and incubated over-night (2 hours pre-incubation with Akt activator SC79 + 16 hours with other stimulants). After this period, supernatants were collected and TNFα levels were measured by ELISA. Abcg1-/- macrophages stimulated with LPS/IFNγ for M1 polarization displayed increased TNFα production compared to WT macrophages as expected (36) (Figure 14). However, after pre-treatment with the Akt activator, TNFα production of Abcg1-/- and WT macrophages were comparable (Figure 14).

Overall, our results suggest that M1 bias of Abcg1-/- macrophages may be mediated through the Akt signaling pathway.

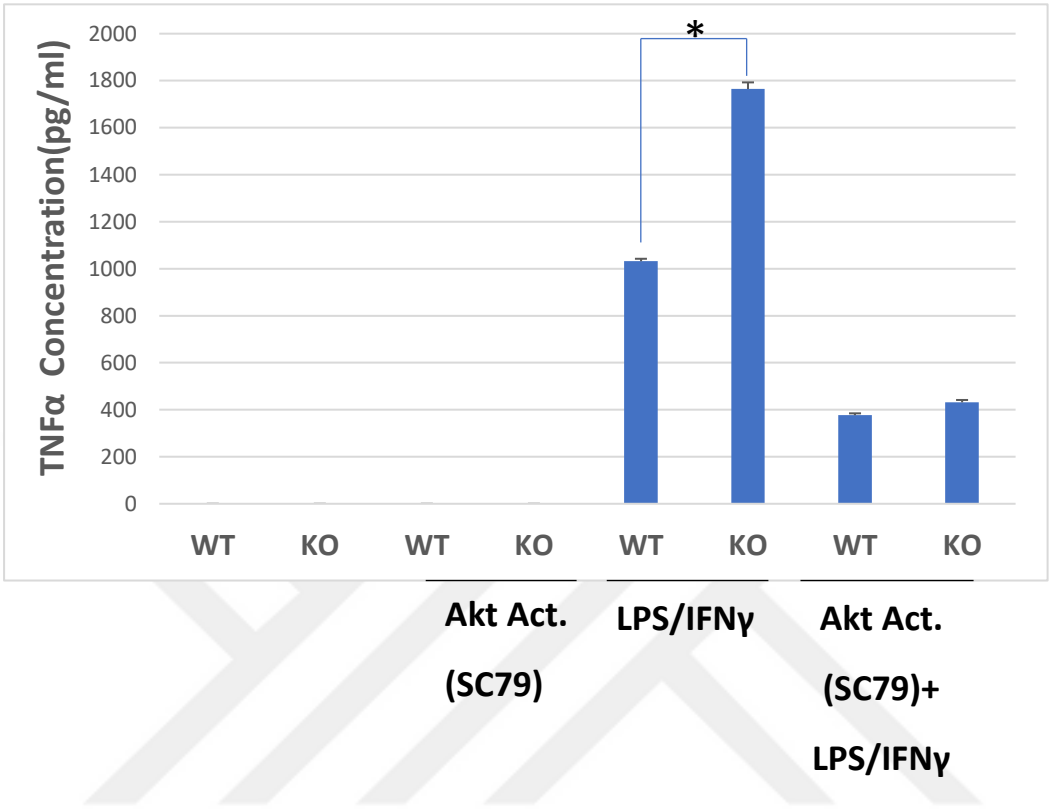


Figure 14. TNFα production in M1 polarized WT and ABCG1 -/- macrophages. WT and ABCG1 -/- BMDMs were pre-treated with the Akt activator (SC79) for left untreated. 2 hours later the cells were stimulated with LPS+IFNγ for M1 polarization overnight. Student's t-test *P<0.05

5. DISCUSSION

Macrophages are innate immune cells that have role in homeostasis and apoptotic cell clearance (10,12). Macrophages polarize to M1 phenotype according to the pathogen derived or environmental stimuli through toll- like receptor (TLR) induction, and M2 polarization occurs by stimulation with IL-4/IL-13 (69). Macrophages also have different roles in tumor microenvironment. M2 macrophages are the most dominant macrophages in the tumor microenvironment, and they support tumor growth (70). Furthermore, M1 macrophages are related with Th1 responses, and they kill pathogens and tumor cells (71). Macrophages also have a role in metastasis and immune suppression in the tumor microenvironment, and increase tumor invasion (72).

ATP-binding transporter family proteins export different molecules across the membrane in eukaryotes, and they are named from A to G (ABCA-ABCG)(23). ABCG1 is highly expressed in many immune cells including macrophages (25). ABCG1 plays a role in reverse cholesterol transport (RCT)(73). It has been shown that ABCG1 is responsible for cholesterol efflux through HDL molecules and has a major role on macrophage cholesterol efflux(73).

Our previous study reported that the tumor growth was suppressed in ABCG1-/- mice in vivo. In the absence of ABCG1, macrophages shift towards an M1 phenotype in the tumor and these M1 macrophages were the main cell type that was responsible for this suppression (36). The aim of this study is to elucidate the underlying mechanism of this M1 switch in ABCG1-/- macrophages. Our first candidate was the AMP- activated protein kinase (AMPK). AMPK is a serine-threonine kinase and it regulates energy homeostasis in the cell (37). It has been shown that AMPK has an important role in inflammation in macrophages (48). Inhibition of AMPK shifts the phenotype of macrophages to a pro-inflammatory M1, while activation of AMPK promotes macrophage polarization through an anti-inflammatory M2 phenotype. (48). In the light of these studies to examine the M1 shift in Abcg1-/- macrophages , we stimulated bone marrow derived macrophages from WT and ABCG1-/- mice with either LPS+IFNγ for M1 polarization or with IL-4 for M2 polarization. From our previous

studies, we know that ABCG1-/- macrophages shift towards M1 and AMPK inhibition promotes M1 polarization in macrophages. Hence, we were expecting to see reduced AMPK phosphorylation/activation in Abcg1-/- macrophages. However, our results show that, in contrary to what we expected, Abcg1-/- macrophages have higher AMPK activation at basal level. Since Abcg1-/- macrophages have increased pro-inflammatory response, increased basal level of AMPK activation in these cells may be a feedback mechanism and AMPK may act as a brake to prevent further increase in inflammation.

In addition, our results demonstrate that M1 stimulus brings down p-AMPK level of Abcg1-/- macrophages at a level lower than WT at 60' and at later time points the difference gradually returned to a similar level as basal. The decrease in AMPK activation 60' after M1 stimulus may explain the increased pro-inflammatory response in Abcg1-/- macrophages.

Akt plays a role in regulation of inflammatory response of macrophages and it acts as a downstream target of AMPK(48). Akt1 activation promotes M2 polarization, while Akt2 activation promotes M1 polarization. Our results show a positive correlation between ABCG1 and Akt1 activation in bone marrow derived macrophages. Moreover, we show that the pre-treatment of macrophages with the Akt activator SC79 decrease TNFα production and reverts the phenotype of Abcg1-/- macrophages to WT. Our data suggest that the reduced activation of Akt1 in Abcg1-/- macrophages could explain the observed M1 bias in these cells. Parallel to our study, a very recent study has shown that when membrane cholesterol level is decreased it provides anti-inflammatory macrophage activation and abolishes IFNγ signaling via Akt activation(74).

Our finding not only provides mechanism for the M1 bias of macrophages in the absence of ABCG1, but also deepens our mechanistic understanding of the M1/M2 switch in macrophages. Therefore, it has the potential to open up new immunotherapeutic approaches for the treatment of cancer.

6. REFERENCES

1. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. J Clin Invest. 2012 Mar;122(3):787-95.
2. Minutti CM, Knipper JA, Allen JE, Zaiss DM. Tissue-specific contribution of macrophages to wound healing. Semin Cell Dev Biol. 2017 Jan;61:3-11.
3. Zhao Y, Zou W, Du J, Zhao Y. The origins and homeostasis of monocytes and tissue-resident macrophages in physiological situation. J Cell Physiol. 2018 Oct;233(10):6425-6439.
4. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. Nature. 2013 Apr 25;496(7446):445-55.
5. Ginhoux F, Jung S. Monocytes and macrophages: developmental pathways and tissue homeostasis. Nat Rev Immunol. 2014 Jun;14(6):392-404.
6. Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. Annu Rev Immunol. 1999;17:593-623.
7. Hirayama D, Iida T, Nakase H. The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. Int J Mol Sci. 2017 Dec 29;19(1).
8. Murray PJ. Macrophage Polarization. Annu Rev Physiol. 2017 Feb 10;79:541-566.
9. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T, Locati M, Mantovani A, Martinez FO, Mege JL, Mosser DM, Natoli G, Saeij JP, Schultze JL, Shirey KA, Sica A, Suttles J, Udalova I, van Ginderachter JA, Vogel SN, Wynn TA. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity. 2014 Jul 17;41(1):14-20.
10. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. Front Biosci. 2008 Jan 1;13:453-61.
11. Krzyszczyk P, Schloss R, Palmer A, Berthiaume F. The Role of Macrophages in Acute and Chronic Wound Healing and Interventions to Promote Pro-wound Healing Phenotypes. Front Physiol. 2018 May 1;9:419.

12. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. Science. 2010 Feb 5;327(5966):656-61.
13. Biswas SK, Chittezhath M, Shalova IN, Lim JY. Macrophage polarization and plasticity in health and disease. Immunol Res. 2012 Sep;53(1-3):11-24.
14. Sica A, Invernizzi P, Mantovani A. Macrophage plasticity and polarization in liver homeostasis and pathology. Hepatology. 2014 May;59(5):2034-42.
15. Mosser DM. The many faces of macrophage activation. J Leukoc Biol. 2003 Feb;73(2):209-12.
16. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 2004 Dec;25(12):677-86.
17. Jetten N, Verbruggen S, Gijbels MJ, Post MJ, De Winther MP, Donners MM. Anti-inflammatory M2, but not pro-inflammatory M1 macrophages promote angiogenesis in vivo. Angiogenesis. 2014 Jan;17(1):109-18
18. Yang M, McKay D, Pollard JW, Lewis CE. Diverse Functions of Macrophages in Different Tumor Microenvironments. Cancer Res. 2018 Oct 1;78(19):5492-5503.
19. Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-associated macrophages as treatment targets in oncology. Nat Rev Clin Oncol. 2017 Jul;14(7):399-416.
20. Rhee I. Diverse macrophages polarization in tumor microenvironment. Arch Pharm Res. 2016 Nov;39(11):1588-1596.
21. Sica A, Schioppa T, Mantovani A, Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. Eur J Cancer. 2006 Apr;42(6):717-27.
22. Borst P, Elferink RO. Mammalian ABC transporters in health and disease. Annu Rev Biochem. 2002;71:537-92.
23. Stefková J, Poledne R, Hubácek JA. ATP-binding cassette (ABC) transporters in human metabolism and diseases. Physiol Res. 2004;53(3):235-43.

24. Fletcher JI, Haber M, Henderson MJ, Norris MD. ABC transporters in cancer: more than just drug efflux pumps. Nat Rev Cancer. 2010 Feb;10(2):147-56.
25. Tarling EJ, Edwards PA. Dancing with the sterols: critical roles for ABCG1, ABCA1, miRNAs, and nuclear and cell surface receptors in controlling cellular sterol homeostasis. Biochim Biophys Acta. 2012 Mar;1821(3):386-95.
26. Tall AR, Yvan-Charvet L, Terasaka N, Pagler T, Wang N. HDL, ABC transporters, and cholesterol efflux: implications for the treatment of atherosclerosis. Cell Metab. 2008 May;7(5):365-75.
27. Kober AC, Manavalan APC, Tam-Amersdorfer C, Holmér A, Saeed A, Fanaee-Danesh E1, Zandl M, Albrecher NM, Björkhem I, Kostner GM, Dahlbäck B, Panzenboeck U. Implications of cerebrovascular ATP-binding cassette transporter G1 (ABCG1) and apolipoprotein M in cholesterol transport at the blood-brain barrier. Biochim Biophys Acta Mol Cell Biol Lipids. 2017 Jun;1862(6):573-588.
28. Wang X, Collins HL, Ranalletta M, Fuki IV, Billheimer JT, Rothblat GH, Tall AR, Rader DJ. Macrophage ABCA1 and ABCG1, but not SR-BI, promote macrophage reverse cholesterol transport in vivo. J Clin Invest. 2007 Aug;117(8):2216-24.
29. Cao XJ, Zhang MJ, Zhang LL, Yu K1, Xiang Y, Ding X, Fan J, Li JC, Wang QS. TLR4 mediates high-fat diet induced physiological changes in mice via attenuating PPARγ/ABCG1 signaling pathway. Biochem Biophys Res Commun. 2018 Sep 10;503(3):1356-1363.
30. Wang N, Ranalletta M, Matsuura F, Peng F, Tall AR. LXR-induced redistribution of ABCG1 to plasma membrane in macrophages enhances cholesterol mass efflux to HDL. Arterioscler Thromb Vasc Biol. 2006 Jun;26(6):1310-6.
31. Kennedy MA, Barrera GC, Nakamura K, Baldán A, Tarr P, Fishbein MC, Frank J, Francone OL, Edwards PA. ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. Cell Metab. 2005 Feb;1(2):121-31.
32. Tarling EJ, Bojanic DD, Tangirala RK, Wang X, Lovgren-Sandblom A, Lusis AJ, Bjorkhem I, Edwards PA. Impaired development of atherosclerosis in Abcg1-/-Apoe-/- mice: identification of specific oxysterols that both accumulate in

Abcg1-/- Apoe-/- tissues and induce apoptosis. Arterioscler Thromb Vasc Biol. 2010 Jun;30(6):1174-80.

33. Lise M. Hardy, Eric Frisdal, and Wilfried Le Goff. Critical Role of the Human ATP-Binding Cassette G1 Transporter in Cardiometabolic Diseases. Int J Mol Sci. 2017 Sep; 18(9): 1892.
34. Baldán A, Gomes AV, Ping P, Edwards PA. Loss of ABCG1 results in chronic pulmonary inflammation. J Immunol. 2008 Mar 1;180(5):3560-8.
35. Wojcik AJ1, Skaflen MD, Srinivasan S, Hedrick CC. A critical role for ABCG1 in macrophage inflammation and lung homeostasis. J Immunol. 2008 Mar 15;180(6):4273-82.
36. Sag D, Cekic C, Wu R, Linden J, Hedrick CC. The cholesterol transporter ABCG1 links cholesterol homeostasis and tumour immunity. Nat Commun. 2015 Feb 27;6:6354.
37. Carling D. The AMP-activated protein kinase cascade--a unifying system for energy control. Trends Biochem Sci. 2004 Jan;29(1):18-24.
38. Carroll KC, Viollet B, Suttles J. AMPKα1 deficiency amplifies proinflammatory myeloid APC activity and CD40 signaling. J Leukoc Biol. 2013 Dec;94(6):1113-21.
39. Sanders MJ, Grondin PO, Hegarty BD, Snowden MA, Carling D. Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. Biochem J. 2007 Apr 1;403(1):139-48.
40. Hawley SA, Davison M, Woods A, Davies SP, Beri RK, Carling D, Hardie DG. Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. J Biol Chem. 1996 Nov 1;271(44):27879-87.
41. Salminen A, Hyttinen JM, Kaarniranta K. AMP-activated protein kinase inhibits NF-κB signaling and inflammation: impact on healthspan and lifespan. J Mol Med (Berl). 2011 Jul;89(7):667-76.
42. Oakhill JS, Scott JW, Kemp BE. AMPK functions as an adenylate charge-regulated protein kinase. Trends Endocrinol Metab. 2012 Mar;23(3):125-32.

43. Racioppi L, Means AR. Calcium/calmodulin-dependent protein kinase kinase 2: roles in signaling and pathophysiology. J Biol Chem. 2012 Sep 14;287(38):31658-65.
44. Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, Frenguelli BG, Hardie DG. Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. Cell Metab. 2005 Jul;2(1):9-19.
45. Chen B, Zhu H. AMPK: A Bridge between Inflammation and Metabolism. JSM Atheroscler. (2016) 1(2): 1008.
46. Corton JM, Gillespie JG, Hawley SA, Hardie DG. 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? Eur J Biochem. 1995 Apr 15;229(2):558-65.
47. Kirchner J, Brüne B, Namgaladze D. AICAR inhibits NFκB DNA binding independently of AMPK to attenuate LPS-triggered inflammatory responses in human macrophages. Sci Rep. 2018 May 17;8(1):7801.
48. Duygu Sag, David Carling, Robert D. Stout, and Jill Suttles. AMP-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. J Immunol. 2008 Dec 15; 181(12): 8633–8641.
49. Yang Z, Kahn BB, Shi H, Xue BZ. Macrophage alpha1 AMP-activated protein kinase (alpha1AMPK) antagonizes fatty acid-induced inflammation through SIRT1. J Biol Chem. 2010 Jun 18;285(25):19051-9.
50. Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal. 2001 Feb;13(2):85-94.
51. Huang BP, Lin CH, Chen HM, Lin JT, Cheng YF, Kao SH. AMPK activation inhibits expression of proinflammatory mediators through downregulation of PI3K/p38 MAPK and NF-κB signaling in murine macrophages. DNA Cell Biol. 2015 Feb;34(2):133-41.
52. Li D, Zhang Y, Ma J, Ling W, Xia M. Adenosine monophosphate activated protein kinase regulates ABCG1-mediated oxysterol efflux from endothelial cells and protects against hypercholesterolemia-induced endothelial dysfunction. Arterioscler Thromb Vasc Biol. 2010 Jul;30(7):1354-62.

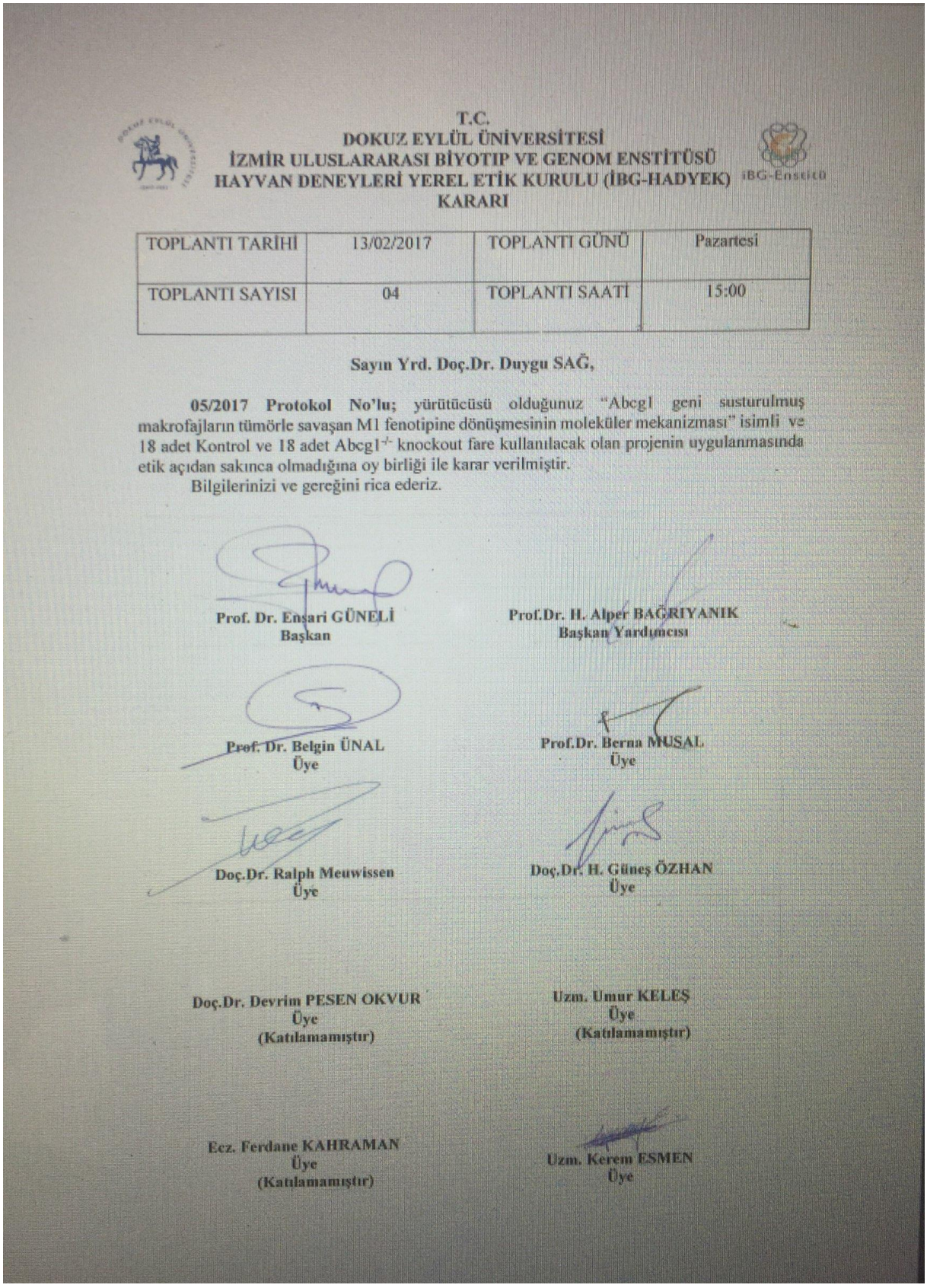
53. Li D, Wang D, Wang Y, Ling W, Feng X, Xia M. Adenosine monophosphate-activated protein kinase induces cholesterol efflux from macrophage-derived foam cells and alleviates atherosclerosis in apolipoprotein E-deficient mice. J Biol Chem. 2010 Oct 22;285(43):33499-509.
54. Lee YG, Lee J, Byeon SE, Yoo DS, Kim MH, Lee SY, Cho JY. Functional role of Akt in macrophage-mediated innate immunity. Front Biosci (Landmark Ed). 2011 Jan 1;16:517-30.
55. Yang ZZ, Tschopp O, Baudry A, Dümmler B, Hynx D, Hemmings BA. Physiological functions of protein kinase B/Akt. Biochem Soc Trans. 2004 Apr;32(Pt 2):350-4.
56. Lee YG, Lee J, Byeon SE, Yoo DS, Kim MH, Lee SY, Cho JY. Functional role of Akt in macrophage-mediated innate immunity. Front Biosci (Landmark Ed). 2011 Jan 1;16:517-30.
57. Koseoglu S, Lu Z, Kumar C, Kirschmeier P, Zou J. AKT1, AKT2 and AKT3-dependent cell survival is cell line-specific and knockdown of all three isoforms selectively induces apoptosis in 20 human tumor cell lines. Cancer Biol Ther. 2007 May;6(5):755-62.
58. Manning BD, Toker A. AKT/PKB Signaling: Navigating the Network. Cell. 2017 Apr 20;169(3):381-405.
59. Scheid MP, Woodgett JR. Unravelling the activation mechanisms of protein kinase B/Akt. FEBS Lett. 2003 Jul 3;546(1):108-12.
60. Huang J, Manning BD. A complex interplay between Akt, TSC2 and the two mTOR complexes. Biochem Soc Trans. 2009 Feb;37(Pt 1):217-22.
61. Altomare DA, Khaled AR. Homeostasis and the importance for a balance between AKT/mTOR activity and intracellular signaling. Curr Med Chem. 2012;19(22):3748-62.
62. Hermida MA, Dinesh Kumar J, Leslie NR. GSK3 and its interactions with the PI3K/AKT/mTOR signalling network. Adv Biol Regul. 2017 Aug;65:5-15.
63. Manning BD, Toker A. AKT/PKB Signaling: Navigating the Network. Cell. 2017 Apr 20;169(3):381-405.

64. Arranz A, Doxaki C, Vergadi E, Martinez de la Torre Y, Vaporidi K, Lagoudaki ED, Ieronymaki E, Androulidaki A, Venihaki M, Margioris AN, Stathopoulos EN, Tsichlis PN, Tsatsanis C. Akt1 and Akt2 protein kinases differentially contribute to macrophage polarization. Proc Natl Acad Sci U S A. 2012 Jun 12;109(24):9517-22.
65. López-Peláez M, Soria-Castro I, Boscá L, Fernández M, Alemany S. Cot/tpl2 activity is required for TLR-induced activation of the Akt p70 S6k pathway in macrophages: Implications for NO synthase 2 expression. Eur J Immunol. 2011 Jun;41(6):1733-41.
66. Luyendyk JP, Schabbauer GA, Tencati M, Holscher T, Pawlinski R, Mackman N. Genetic analysis of the role of the PI3K-Akt pathway in lipopolysaccharide-induced cytokine and tissue factor gene expression in monocytes/macrophages. J Immunol. 2008 Mar 15;180(6):4218-26.
67. Rückerl D, Jenkins SJ, Laqtom NN, Gallagher IJ, Sutherland TE, Duncan S, Buck AH, Allen JE. Induction of IL-4Rα-dependent microRNAs identifies PI3K/Akt signaling as essential for IL-4-driven murine macrophage proliferation in vivo. Blood. 2012 Sep 13;120(11):2307-16.
68. Zhu YP, Brown JR1, Sag D, Zhang L, Suttles J. Adenosine 5'-monophosphate-activated protein kinase regulates IL-10-mediated anti-inflammatory signaling pathways in macrophages. J Immunol. 2015 Jan 15;194(2):584-94.
69. Zhou D, Huang C, Lin Z, Zhan S, Kong L, Fang C, Li J. Macrophage polarization and function with emphasis on the evolving roles of coordinated regulation of cellular signaling pathways. Cell Signal. 2014 Feb;26(2):192-7.
70. Hao NB, Lü MH, Fan YH, Cao YL, Zhang ZR, Yang SM. Macrophages in tumor microenvironments and the progression of tumors. Clin Dev Immunol. 2012;2012:948098.
71. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 2004 Dec;25(12):677-86.
72. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell. 2006 Jan 27;124(2):263-6.

73. Vaughan AM, Oram JF. ABCA1 and ABCG1 or ABCG4 act sequentially to remove cellular cholesterol and generate cholesterol-rich HDL. J Lipid Res. 2006 Nov;47(11):2433-43.
74. Goossens P, Rodriguez-Vita J, Etzerodt A, Masse M, Rastoin O, Gouirand V, Ulas T, Papantonopoulou O, Van Eck M, Auphan-Anezin N, Bebien M, Verthuy C, Vu Manh TP, Turner M, Dalod M, Schultze JL, Lawrence T. Membrane Cholesterol Efflux Drives Tumor-Associated Macrophage Reprogramming and Tumor Progression. Cell Metab. 2019 Jun 4;29(6):1376-1389.e4.

7. APPENDIX

7.1. Research Ethics Committee Approval



T.C.
DOKUZ EYLÜL ÜNİVERSİTESİ
İZMİR ULUSLARARASI BİYOTIP VE GENOM ENSTİTÜSÜ
HAYVAN DENEYLERİ YEREL ETİK KURULU (İBG-HADYEK)
KARARI

TOPLANTI TARİHİ	13/02/2017	TOPLANTI GÜNÜ	Pazartesi
TOPLANTI SAYISI	04	TOPLANTI SAATİ	15:00

Sayın Yrd. Doç.Dr. Duygu SAĞ,

05/2017 Protokol No'lu; yürütücüsü olduğunuz "Abcg1 geni susturulmuş makrofajların tümörle savaşan M1 fenotipine dönüşmesinin moleküler mekanizması" isimli ve 18 adet Kontrol ve 18 adet Abcg1$^{-/-}$ knockout fare kullanılacak olan projenin uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

Prof. Dr. Ensari GÜNELİ
Başkan

Prof.Dr. H. Alper BAĞRIYANIK
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Prof. Dr. Belgin ÜNAL
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Doç.Dr. Devrim PESEN OKVUR
Üye
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EDUCATION

2016- Present	Izmir International Biomedicine and Genome Institute/ Molecular Biology and Genetics (MSc)
2011- 2015	Gebze Technical University/ Molecular Biology and Genetics (BSc)

INTERNSHIPS

July - September	**2014**	Instituto Gulbenkian de Ciencia /Principles of Nuclear Division Lab(Summer Internship).
June - July	**2014**	Hacettepe University/ Stem Cell Research and Application Center (Summer Internship).

PRESENTATIONS AT CONFERENCES

May	**2019**	The cholesterol transporter ABCG1 modulates macrophage polarization in human monocyte-derived macrophages/ Poster Presentation/ Annual Meeting of The American Association of Immunologists (AAI) -IMMUNOLOGY 2019^{TM}- San Diago/USA
April	**2019**	Molecular mechanism for M1 bias of ABCG1-deficient Macrophages/ International Molecular Immunology & Immunogenetics Congress IV (MIMIC IV)/ Oral Presentation/ Bursa /Turkey
September	**2018**	Molecular mechanism for M1 bias of ABCG1-deficient macrophages/ European Congress of Immunology(ECI)- Poster Presentation- Amsterdam/ Netherlands

CONGRESSES AND COURSES

December 2017	Therapeutic Engineering and Production Workshop/ Dokuz Eylül University
December 2017	New Frontiers in Life Sciences/ Dokuz Eylül University
April 2017	24th National Immunology Congress / Turkish Society of Immunology
March 2017	Animal Handling Course / Dokuz Eylül University
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